Assembly, Trafficking and Downstream Signaling of α5-GABA$_A$ Receptors

Dissertation

zur

Erlangung der naturwissenschaftlichen Doktorwürde

(Dr. sc. nat.)

vorgelegt der

Mathematisch-naturwissenschaftlichen Fakultät

der

Universität Zürich

von

Ela Balic

aus Kroatien

Promotionskomitee

Prof. Dr. Raimund Dutzler

Prof. Dr. Jean-Marc Fritschy

Dr. Dietmar Benke

Zürich, 2007

True insight comes from within.

(Jostein Gaarder, "Sophie's World – Socrates")

To my parents.

Table of contents

1 Zusammenfassung

$GABA_A$-Rezeptoren sind pentamere Liganden-gesteuerte Ionenkanäle, die die tonisch-inhibitorische Transmission im Gehirn vermitteln. Neben der Bindungstelle für den Neurotransmitter GABA verfügen $GABA_A$-Rezeptoren über verschiedene distinkte allosterische Bindungsstellen, welche u. a. die klinisch relevanten Wirkungen von Benzodiazepinen, Barbituraten und einigen Anästhetika mediieren. $GABA_A$-Rezeptoren treten in einer Vielzahl von Subtypen auf. Die häufigsten und klinisch relevanten Rezeptorsubtypen setzen sich aus zwei α- (α1, 2, 3 oder 5), zwei β (β1, 2 oder 3) und der γ2-Untereinheit zusammen. Die einzelnen Rezeptorsubtypen weisen unterschiedliche temporale und regionale Expressionsmuster auf, was auf spezialisierte physiologische Funktionen hinweist. Die vorliegende Studie konzentrierte sich auf die α5-$GABA_A$-Rezeptoren, die in vergleichsweise hoher Konzentration in den Pyramidenzellen des Hippocampus exprimiert werden. Sie sind vor allem in extrasynaptischen somato-dendritischen Bereichen lokalisiert und spielen eine wichtige Rolle in der Regulation von Lern- und Gedächtnisprozessen.

Unerwarteter Weise führte eine Punkmutation in der Benzodiazepin-Bindungsstelle der α5-Untereinheit (H105R), die in das Mausgenom eingeführt wurde um eine Unempfindlichkeit der α5-$GABA_A$-Rezeptoren gegenüber Diazepam zu erreichen, zu einer verringerten Expression der α5-Untereinheit im Hippocampus. Diese Reduktion der α5-Untereinheit korrelierte mit einer verstärkten Angstkonditionierung, die eine Hippocampus-abhängige Form des Assoziantions-lernens darstellt (Crestani *et al*. 2002). Da die Ursache für Reduktion der α5-Untereinheit im Hippocampus unbekannt war, wurde in der vorliegenden Studie untersucht, ob die H105R-Mutation das Targeting und/oder die Assemblierung der α5-$GABA_A$-Rezeptoren beeinflusst. Darüber hinaus wurde analysiert, ob die verminderte Expression der α5-Untereinheit einen Einfluss auf die Expression und/oder die Aktivität von Signalproteinen hat, die eine wichtige Funktion bei Lern- und Gedächtnisprozessen ausüben.

Eine Western-Blot-Analyse zeigte, dass die Expression des α5-Untereinheitenproteins in α5(H105R)-Mäusen nur moderat (~23%) reduziert ist. Allerdings wiesen Radioligand-Bindungsstudien auf eine starke Reduktion (~50-70%) der α5-$GABA_A$ Rezeptoren hin. Immunhistochemische sowie ELISA Experimente ergaben, dass sowohl

CREB eine adaptive Kompensation einer chronisch erhöhten NMDA Rezeptor Aktivität darstellen.

2 Summary

$GABA_A$ receptors are pentameric ligand-gated ion channels that are major mediators of the inhibitory tone throughout the central nervous system. They are the site of action of many clinically important drugs, including benzodiazepines, barbiturates and some general anesthetics. $GABA_A$ receptors exhibit an enormous structural heterogeneity based on the combinatorial assembly of a variety of distinct subunits. The major and clinically relevant $GABA_A$ receptor subtypes are composed of α1,2,3,5, β1-3 and γ2 subunits. These receptor subtypes display distinct temporal and spatial expression patterns, indicating different specialized physiological roles. In this study we focused on α5-$GABA_A$ receptors, which are highly expressed in hippocampal pyramidal cells. They are predominantly localized extrasynaptically in somato-dendritic membranes and play an important role in regulating learning and memory.

Introduction of the H105R point mutation into the α5 subunit, to render α5-containing receptors insensitive to the clinically important benzodiazepine site drug diazepam, unexpectedly resulted in a reduced level of α5 subunit protein in the hippocampus. This reduction in α5 subunit protein was associated with facilitated trace fear conditioning, a hippocampus dependent form of associative learning (Crestani *et al.* 2002). Since the cause for the reduction of α5-subunit protein is unknown, we aimed in the present study at exploring whether the α5(H105R) mutation affects the targeting and/or the assembly of α5-$GABA_A$ receptors. In addition, we analyzed the effect of diminished hippocampal α5-$GABA_A$ receptors on the activity of downstream signaling proteins important for learning and memory.

Western blot analysis revealed that the level of the α5 subunit protein is only moderately reduced (~23%) in α5(H105R) mice. However, when the level of receptors was determined by radioligand binding, α5-$GABA_A$ receptors were found to be severely decreased (~50-70%) not only in the hippocampus but in all brain areas expressing α5-$GABA_A$ receptors. Immunocytochemical and ELISA experiments on α5-$GABA_A$ receptors expressed in HEK293 cells demonstrated similar expression levels and cell surface targeting irrespective of whether wild type or mutant α5-$GABA_A$ receptors were used. Likewise, expression of GFP-tagged α5 subunits in cultured cortical neurons indicated that the H105R mutation does not appreciably affect expression and targeting

of α5(H105R)-$GABA_A$ receptors in neurons. These results suggest that overexpressing α5(H105R) subunits in both HEK293 cells and neurons may override subtle impairments that lead to the partial loss of α5-$GABA_A$ receptors observed in α5(H105R) mice.

In vivo, two different α subunits are frequently present in $GABA_A$ receptors e. g. α1 and α5. The subunit positioning defines the drug binding properties of $GABA_A$ receptors containing two different types of α subunits in a single receptor complex. Analysis by sucrose density gradient centrifugation and radioligand binding revealed a large fraction of misassembled α5(H105R)-$GABA_A$ receptors as indicated by their inability to bind ligands of the benzodiazepine site. In addition, in those α5(H105R)-$GABA_A$ receptors that assemble into ligand binding receptors the positioning of the α5(H105R) subunit in receptor complexes containing two distinct types of α subunits, e. g. α1 and α5, appeared to be changed. These findings imply an important role of histidine 105 in determining the position of the α5 subunit within the receptor complex

Finally, the effect of a diminished α5-$GABA_A$ receptor expression on the activity of downstream signaling proteins important for learning and memory, which are under the control of NMDA receptors, was analyzed in the hippocampus. By Western blot analysis, reduced phosphorylation levels, i. e. activation states, selectively for CaMKII, MAPK p44/42 and CREB, with no change in their protein levels, were observed in α5(H105R) mice. Since a reduced expression of functional α5-$GABA_A$ receptors in the hippocampus is expected to result in a reduced inhibitory tone at spines of hippocampal pyramidal neurons and thus to an increased NMDA receptor activity, diminished activity of CaMKII, p44/42 MAPK and CREB may compensate for a chronically enhanced NMDA receptor response and thus may represent an adaptive response.

3 Introduction

The central nervous system operates by a fine-tuned balance between excitatory and inhibitory signaling. The most abundant inhibitory neurotransmitter is GABA, which acts at ionotropic $GABA_A$ and metabotropic $GABA_B$ receptors (reviewed by Bormann 2000). GABAergic function is modulated on several levels, including transmitter synthesis by two isoforms of glutamic acid decarboxylase (GAD) (Soghomonian and Martin 1998), vesicular storage (Dumoulin *et al*. 1999), Ca^{2+}-dependent and independent release (Wall and Usowicz 1997), re-uptake in neurons and glial cells (Borden 1996, Quick *et al*. 1997) as well as activation of multiple receptors localized pre-, post- and extrasynaptically (Fritschy and Brünig 2003, Lüscher and Keller 2004). The significance of GABAergic inhibition is reflected in multiple neurological and psychiatric diseases such as epilepsy (Olsen *et al*. 1999), anxiety disorders (Malizia *et al*. 1999), ethanol dependence (Morrow *et al*. 2001), Huntington's disease (Kunig *et al*. 2000), Angelman syndrome (DeLorey *et al*. 1998) and schizophrenia (Nutt and Malizia 2001). In addition to being key sites for synaptic inhibition, $GABA_A$ receptors are important drug targets, including benzodiazepines, barbiturates, some general anesthetics and ethanol (Dilger 2002, Mohler *et al*. 2002, Rudolph and Mohler 2006).

3.1 Structure of $GABA_A$ receptors

$GABA_A$ receptors are members of the ligand-gated ion channel superfamily that includes nicotinic acetylcholine receptors (nAChRs), glycine receptors and the serotonin 5-hydroxytryptamine type 3 receptors (5-HT_3R). This family of receptors is activated by presynaptically released ligands, which induce conformational changes in the receptor protein that opens the integral membrane-spanning ion-channel (Fig. 1). This results in an inward or outward flow of ions, depending on the electrochemical gradient of the ion. $GABA_A$ receptors are primarily permeable for chloride ions, although bicarbonate ions can cross the channel pore as well. Structurally, $GABA_A$ receptors are pentameric assemblies constructed from subunits derived from related genes or gene families (McDonald and Olsen 1994). Each subunit contains a large extracellular domain containing the ligand binding sites, four transmembrane spanning domain and a large intracellular loop connecting transmembrane domains three and four (Fig. 1). Subunits that are building blocks for $GABA_A$ receptors involve α(1-6), β(1-3), γ(1-3), δ, ε, π, θ and ρ(1-3) with further variation resulting from alternative splicing (Barnard *et al*.

1998). The combinatorial assembly of these various subunits could lead to an enormous molecular heterogeneity of $GABA_A$ receptor subtypes; however certain subunit combinations are preferred (McKernan & Whiting 1996). The most abundant native receptors are formed from α1, β2 and γ2 subunits (Benke *et al*. 1991, McKernan and Whiting 1996). The likely stoichiometry is 2 α, 2 β and 1 γ subunit arranged around the ion channel (Tretter *et al*. 1997). The δ, ε, π and θ subunits are believed to substitute for the γ subunit in such receptor assemblies (Fig. 1). In addition, receptors with different subunit compositions are distributed to different cellular locations, where they are positioned to mediate primarily synaptic or extrasynaptic signaling (reviewed by Farrant and Nusser 2005, Fritschy and Brunig, 2003).

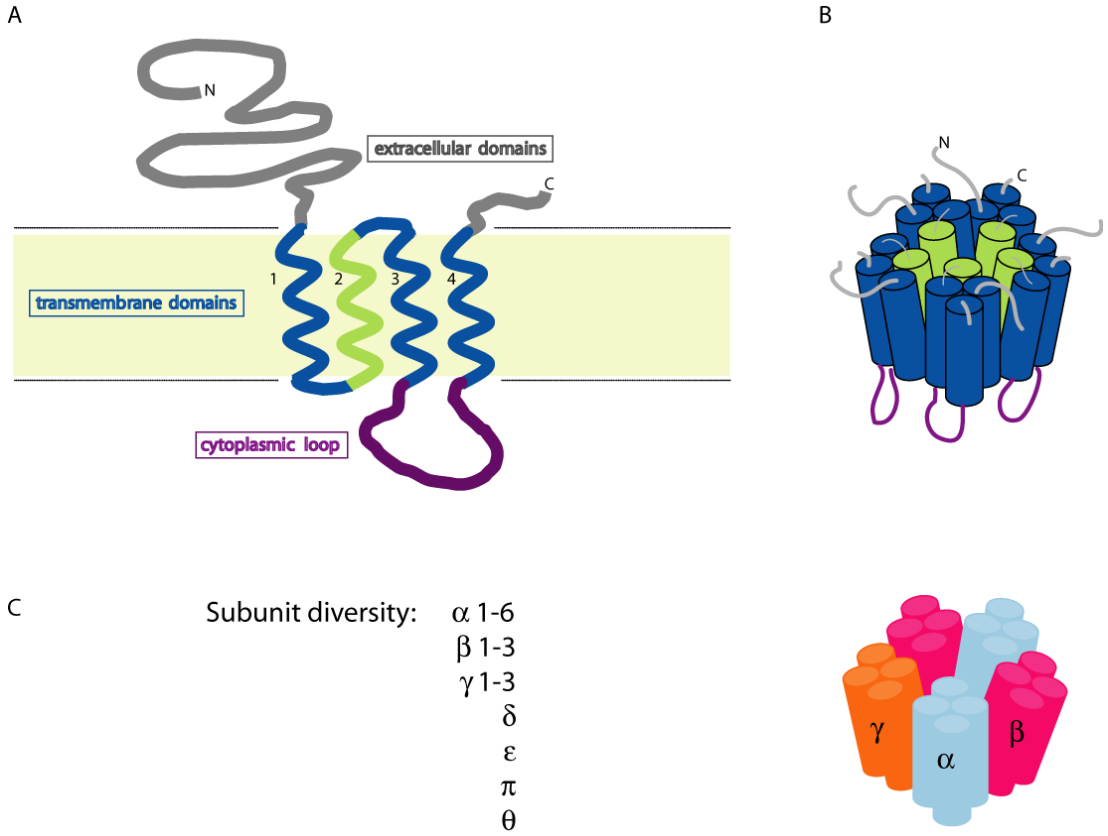

Figure 1. Structure of $GABA_A$ receptor. (A) Each receptor subunit contains four hydrophobic transmembrane (TM) domains. The large N-terminal domain is located extracellularly and incorporates the neurotransmitter binding sites. The intracellular domain (cytoplasmic loop) between TM3 and TM4 is the most divergent part of individual receptor subunits and contains consensus sites for various protein kinases. The transmembrane domain TM2 is believed to form the lining of the ion channel. (B) Proposed pentameric structure of $GABA_A$ receptor. The TM2 domains (in green) are composed of either neutral or positively charged basic residues, which are thought to contribute to the anion selectivity of this channel. (C) Most $GABA_A$ receptors are composed of α, β and γ subunits in stoichiometry of 2:2:1. The δ, ε, θ can replace the γ subunit in some receptor subtypes. *Adapted from Moss and Smart (2001).*

3.2 $GABA_A$ receptor assembly

The pentameric $GABA_A$ receptors are built out of 16 different subunits and theoretically have the potential for an enormous structural diversity (theoretically 165 distinct subtypes). Despite this potential, relatively few functional receptor complexes have been identified *in vivo* (McKernan and Whiting 1996). Limiting receptor diversity could be a consequence of brain-region-specific and temporal expression of different subunits, but many neuronal cell types express simultaneously different subunit subtypes (Wisden and Seeburg 1992, Smith *et al*. 1998). This implies the existence of subcellular mechanisms for differential receptor assembly. There seems to be a combination of the discrete sites for receptor assembly present in each $GABA_A$ receptor subunit (Job and Eberwine 2001). Specific amino acid sequences present in the N-termini of the subunits have been found to control $GABA_A$ receptor subunit oligomerization (reviewed by Kittler *et al*. 2002). A domain in the α1 subunit, which is conserved in all α subunit isoforms (amino acids 58-67), is essential for association of the α1 with the β3 subunit (Taylor *et al*. 2000). Additionally, W69 and W94 have been identified to play an important role in the assembly of $GABA_A$ receptors and are common to all subunits of the ligand-gated ion channel family (Srinivasan *et al*. 1999). In the γ2 subunit, the amino acids 83-90 were identified as crucial for the assembly with β3 subunits and amino acids 91-104 for the assembly with α1 subunits (Klausberger *et al*. 2000). Reciprocally, in α1 subunits this corresponded to amino acids 80-100 (Klausberger *et al*. 2001). In addition, alanine 108 in the α1 subunit was found to be important for the formation of assembly intermediates with β3 and γ2 subunits (Sarto-Jackson *et al*. 2006). On the basis of these experimental findings it has been suggested that the α1 subunit contains at least three distinct subunit-binding sites: two binding sites on opposite faces of the N-terminal domain mediate assembly with β3 subunits and on one face there is an additional binding site for γ2 subunits (Klausberger *et al*. 2001). This would allow the α1 subunit to bind either two β3 subunits or one β3 and one γ2 subunit. Furthermore, Klausberger *et al*. (2001) suggest that the single α1-γ2 inter-subunit contact site controls the subunit assembly and stoichiometry of $GABA_A$ receptors.

3.3 $GABA_A$ receptor targeting to the cell surface

For most $GABA_A$ receptor subunits, assembly into hetero-oligomeric complexes facilitates access to the cell surface. Studies on recombinant $GABA_A$ receptors composed of various subunit combinations revealed that access to the cell surface is limited to αβ and αβγ subunit combinations. Single expressed subunits and αγ or βγ combinations are retained within the endoplasmatic reticulum (ER) (reviewed by Kittler *et al*. 2002). Most importantly, αβγ receptors are assembling at the expense of αβ formation when all three subunits are present (Bollan *et al*. 2003).

The quality control for correctly assembled receptors occurs in the ER, where subunits associate with chaperone molecules such as calnexin, BiP (heavy chain binding protein) and protein disulphide isomerase (Connoly *et al*. 1996, Gorrie *et al*. 1997). Calnexin recognizes immature glycans, BiP exposed hydrophobic regions and the isomerase catalyses disulphide bridges formation. Incorrectly assembled receptors are bound to BiP and calnexin and retained in ER via luminal KDEL retention signals within the chaperone proteins until being degraded. Correctly assembled receptors are released from the ER and travel to the cell surface. Retention of $GABA_A$ receptors in the ER may also serve to regulate the availability of particular subunits for assembly and subsequent insertion of new $GABA_A$ receptors into synapses (Kittler *et al*. 2000). Because both the N-terminus and the C-terminus of $GABA_A$ receptor subunits extend outside of the cell membrane, the intracellular loop located between transmembrane spanning domains TM3 and TM4 (Fig. 1) is the most important domain interacting with proteins involved in regulating the synaptic localization and intracellular trafficking (Fig. 2). These proteins have been shown to play important roles in modulating the activities of $GABA_A$ receptors ranging from enhancing trafficking (GABARAP) to stabilizing surface (Plic-1) and internalized receptors (HAP1) (reviewed by Chen and Olsen 2007). Associated proteins can influence the surface number of $GABA_A$ receptors (GABARAP, GRIP, BIG2, NSF) as well as guide the receptors to the synaptic (gephyrin) or extrasynaptic sites (radixin) (Kittler and Moss 2003, Chen and Olsen 2007). Accompanied by interacting proteins, the $GABA_A$ receptors traffic along microtubules from intracellular compartments to the plasma membrane (Wang and Olsen 2000), move between synapses and extrasynaptic sites (Thomas *et al*. 2005) and finally internalize from the plasma membrane to the degradation pathway (Kittler *et al*. 2000) (Fig. 2).

mutierte als auch wildtyp α5-$GABA_A$-Rezeptoren nach transienter Expression in HEK293 Zellen ein vergleichbares Expressionsniveau und ein ähnliches Zellöberflächen-Targeting aufwiesen. Darüber hinaus zeigte die Expression von GFP-markierten α5-Untereinheiten in kultivierten Neuronen, dass die H105R-Mutation die Expression und das Targeting der α5(H105R)-$GABA_A$ Rezeptoren nicht spürbar beeinflusst. Diese Resultate deuten darauf hin, dass die Überexpression der α5(H105R)-Untereinheiten in HEK293-Zellen und in Neuronen subtile Beeinträchtigungen verdecken, die zum partiellen Verlust der α5-$GABA_A$-Rezeptoren in den α5(H105R) Mäusen führen.

In vivo sind häufig zwei unterschiedliche α-Untereinheiten in einem $GABA_A$-Rezeptorkomplex anzutreffen (z. B. α1 und α5), wobei die Positionierung der jeweiligen α-Untereinheiten in Bezug zur γ2-Untereinheit die Liganden-Bindungseigenschaften dieser $GABA_A$-Rezeptoren definiert. Eine Analyse mittels Saccharose-Dichtegradienten und Radioligand-Bindungsexperimenten zeigte, dass ein grosser Anteil von α5(H105R)-$GABA_A$ Rezeptoren offensichtlich fehlerhaft assembliert war, da sie Liganden der Benzodiazepin-Bindungsstelle nicht mehr binden konnten. Weiterhin weisen Immunpräzipitations-Experimente darauf hin, dass in Rezeptoren die zwei unterschiedliche α-Untereinheiten enthielten (α1 und α5), die Positionierung der α-Untereinheiten verändert war. Diese Resultate deuten auf eine wichtige Rolle des Histidinrestes 105 der α5-Untereinheit in der korrekten Assemblierung von α5-$GABA_A$-Rezeptoren hin.

Abschliessend wurde der Effekt einer reduzierten α5-$GABA_A$-Rezeptorexpression in den α5(H105R)-Mäusen auf die Expression und Aktivität von Signalproteinen untersucht, die eine wichtige Rolle in NMDA-Rezeptor kontrollierten Lern- und Gedächtnisprozessen spielen. Eine Western-Blot-Analyse ergab einen reduzierten Phosphorylierungszustand, d. h. Aktivierungsgrad, von CaMKII, MAPK p44/42 und CREB in den α5(H105R)-Mäusen bei gleichbleibendem Expressionsniveau dieser Proteine. Eine reduzierte Expression von α5-$GABA_A$-Rezeptoren im Hippocampus führt voraussichtlich zu einem verminderten inhibitorischen Tonus in den Pyramidenzellen und bedingt vermutlich eine erhöhte Aktivität des NMDA Rezeptors. Daher könnte die beobachtete reduzierte Aktivität von CaMKII, MAPK p44/42 und

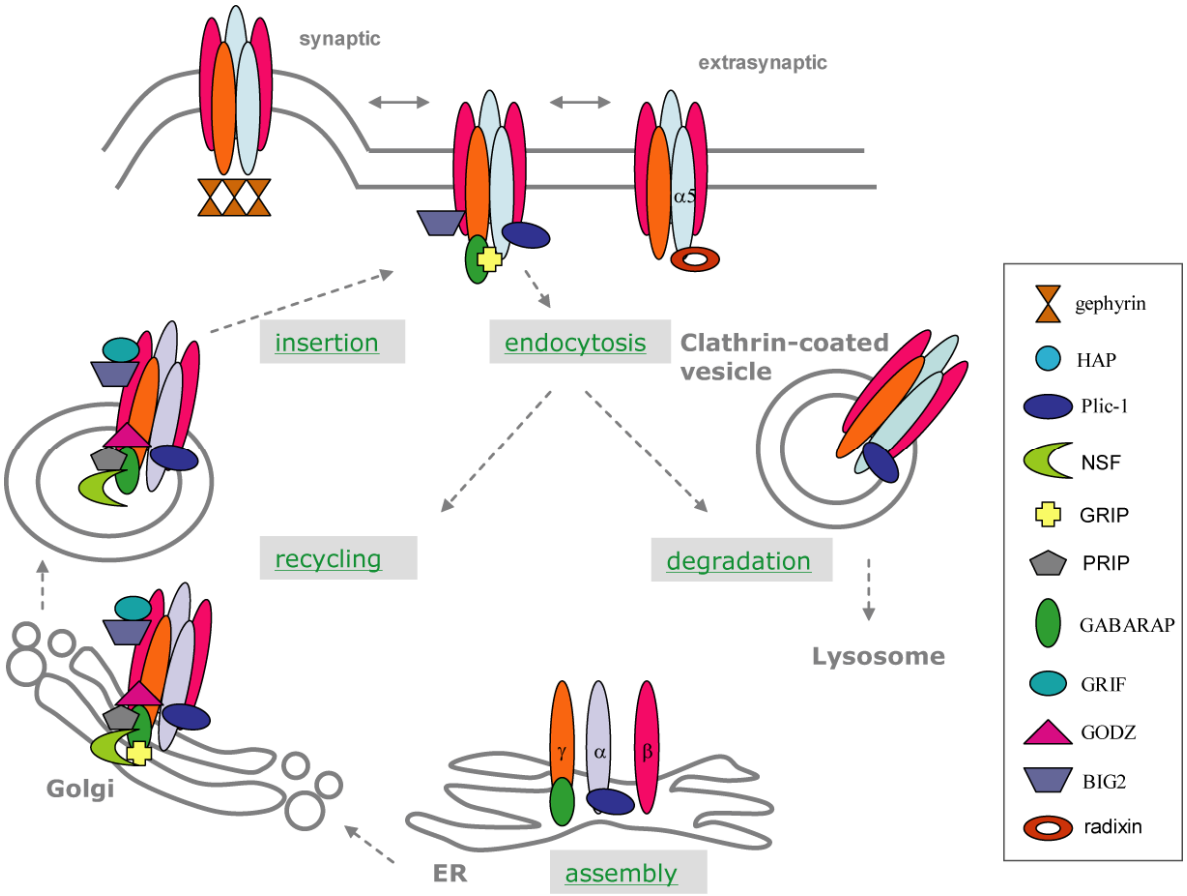

Figure 2. $GABA_A$ receptor trafficking is facilitated by receptor-associated proteins. $GABA_A$ receptors are assembled in the ER and αβ as well as αβγ subunit combinations are able to transit to the Golgi apparatus. Protein interactions in the Golgi apparatus are believed to facilitate intracellular transport of the receptor. At synaptic sites, $GABA_A$ receptors colocalize with the scaffold protein gephyrin, whereas at extrasynaptic sites α5-$GABA_A$ receptors interact with radixin. $GABA_A$ receptors are internalized via clathrin-coated pits. Abbreviations: HAP, Huntingtin-associated protein; Plic-1, proteins that link integrin associated protein to the cytoskeleton; NSF, N-ethylmaleimide-sensitive factor; GRIP, glutamate receptor interacting protein; PRIP, phospholipase C-related inactive proteins; GABARAP, GABA receptor associated protein; GRIF, $GABA_A$ receptor interacting factor; GODZ, Golgi-specific zinc finger protein; BIG2, brefeldin A-inhibited GDP/GTP exchange factor 2. *Adapted from Kittler and Moss (2003) and Chen and Olsen (2007).*

3.4 Pharmacology and distribution of $GABA_A$ receptor subtypes

$GABA_A$ receptors are important drug targets since their malfunction is involved in human diseases such as epilepsy, anxiety disorders, ethanol dependence, Huntington's disease and schizophrenia (reviewed by Mohler 2006). There are several drugs in clinical use, which specifically interact with $GABA_A$ receptors, such as benzodiazepines, barbiturates and some anesthetics (reviewed by Rudolph and Mohler 2006). These compounds are allosteric modulators, which bind to distinct sites on the receptor to increase its activity and therefore enhance inhibitory synaptic transmission. Benzodiazepines are widely used for their anxiolytic, sedative, hypnotic, muscle relaxant and anticonvulsant action (reviewed by Whiting 2003) and moreover have proved to be important research tool for analyzing the functional role of $GABA_A$ receptor subtypes.

The majority of $GABA_A$ receptors are characterized by their sensitivity to the clinically important agonist of the benzodiazepine site, e.g. diazepam. These receptors contain the α1, α2, α3 or α5 subunit in combination with a β and the γ2 subunit. The binding pocket for benzodiazepines is located the interface of the γ2 and α1, α2, α3 or α5 subunits. This position is homologous to the GABA binding site, which is located between α and β subunits (Fig. 3) (Boilleau *et al*. 1999, Teissere and Czajkowski 2001). Interestingly, there is an overlap of the identified assembly signals in the α and γ subunits and the benzodiazepine binding site (Klausberger *et al*. 2000, Sarto *et al*. 2002).

$GABA_A$ receptors that do not respond to agonists of the benzodiazepine site are of low abundance in the brain and characterized mainly by the α4 and α6 subunits. Diazepam sensitivity of $GABA_A$ receptors depends on the presence of a histidine residue in the drug-binding domain of α1, α2, α3 and α5 subunits, whereas α4 and α6 subunits contain at the homologous position an arginine residue (Wieland *et al*. 1992, Benson *et al*. 1998). This feature has been used to analyze the contribution of $GABA_A$ receptor subtypes to the diverse actions of diazepam by generating mouse lines that contain a histidine to arginine point mutation in the α1, α2, α3 and α5 subunits (Rudolph *et al*. 1999, McKernan *et al*. 2000, Löw *et al*. 2000, Crestani *et al*. 2002). These studies have revealed that the sedative action of diazepam is mediated by α1-$GABA_A$ receptors, the

anxiolytic action by α2-$GABA_A$ receptors and muscle relaxant activity partially by α3- as well as by α5-$GABA_A$ receptors (reviewed by Rudolph and Mohler 2004).

The physiological significance of the structural diversity of $GABA_A$ receptors is reflected in differences of their channel kinetics, affinity for GABA, rate of desensitization and interaction with other proteins (reviewed in Mohler 2006). In addition, receptor subtypes differ in their distribution throughout the brain, partially show a cell-type specific expression and when present in the same neuron display very often a domain-specific location (Tab. 1, Fig. 4). $GABA_A$ receptors can be roughly divided into preferentially synaptic types that contain the γ2 subunit and mediate phasic inhibition and extrasynaptic receptor subtypes that contain the δ subunit and contribute selectively to tonic inhibition (reviewed in Farrant and Nusser 2005). However, this rule does not account for α5-containing receptors, which assemble with the γ2 subunit and are mostly localized extrasynaptically were they mediate tonic inhibition (Brünig *et al*. 2002, Crestani *et al*. 2002).

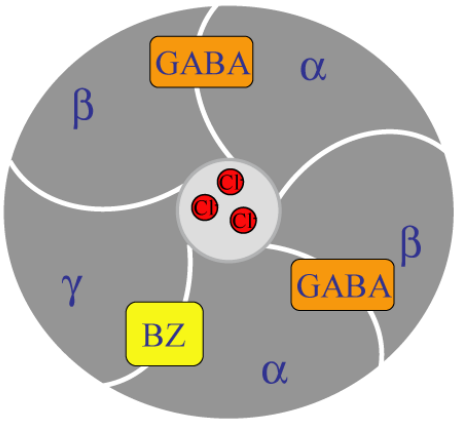

Figure 3. Model of a pentameric $GABA_A$ receptor comprising two α, two β and the γ2 subunit (top-view). The binding sites for GABA and benzodiazepines (BZ) are located at the interface of α/β and α/γ2 subunits, respectively. *Adapted from Ernst et al. (2005).*

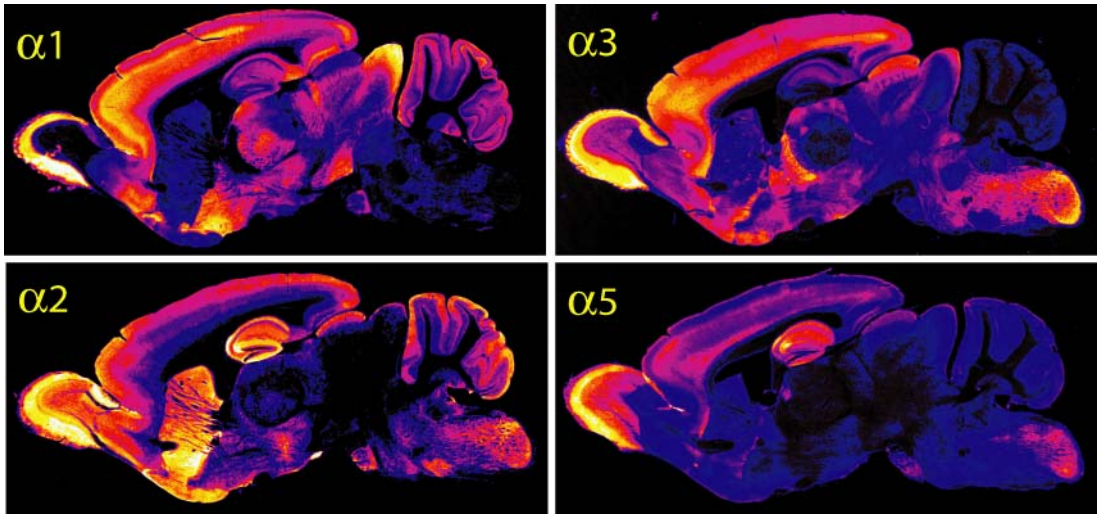

Figure 4. Distribution of $GABA_A$ receptor α subunits. The four classes of diazepam-sensitive $GABA_A$ receptors are distinguished by the type of α-subunit (α1, α2, α3, α5) and their largely distinct neuronal localizations as demonstrated immunohistochemically in mouse brain sections. White color indicates high subunit abundance while blue indicates low subunit abundance. (from Mohler *et al*. 2002).

Table 1. Function and distribution of $GABA_A$ receptor subtypes in the brain *(Mohler 2006)*

Receptor composition	Pharmacological characteristics	Regional and neuronal localization	Subcellular localization
α1β2γ2	Major $GABA_A$ receptor subtype (60 %). Mediates the sedative, amnesic and anticonvulsant action of benzodiazepine site agonists. High affinity for classical benzodiazepines, zolpidem and the antagonist flumazenil.	Cerebral cortex (layer I-VI, selected interneurons and principal cells); hippocampus (selected interneurons and principal cells); pallidum striatum (interneurons); cerebellum (Purkinje and granule cells); deep cerebellar nuclei; amygdala; basal forebrain; substanitia nigra pars reticulate; inferior colliculus; brainstem.	Synaptic (soma and dendrites) and extrasynaptic in all neurons with high expression.
α2β3γ2	Minor subtype (15-20 %). Mediates anxiolytic action of benzodiazepine site agonists. High affinity for classical benzodiazepine agonists and the anatagonist flumazenil. Intermediate affinity for zolpidem.	Cerebral cortex (layers I-IV); hippocampus (principal cells); olfactory bulb (granule cells); striatum (spiny stellate cells); inferior olivary neurons (dendrites); hypothalamus; amygdala (principal cells); superior colliculus; motor neurons.	Mainly synaptic, enriched in axon initial segment of cortical and hippocampal pyramidal cells.
α3βnγ2	Minor subtype (10-15 %). High affinity for classical benzodiazepine agonists and the antagonist flumazenil. Intermediate affinity for zolpidem.	Cerebral cortex (principal cells in layers V and VI, some axon initial segments); hippocampus (some hilar cells); olfactory bulb (tufted cells); thalamic reticular neurons; cerebellum (Golgi type II cells); medullary reticular formation; inferior olivary neurons; amygdala; superior colliculus; brainstem; spinal cord; medial septum; basal forebrain cholinergic neurons; raphe and locus coeruleus (serotoninergic and catecholaminergic neurons).	Mainly synaptic, including some axon initial segments; extrasynaptic in inferior olivary neurons.
α4βnδ	Less than 5 % of all receptors. Insensitive to classical benzodiazepine agonists and zolpidem.	Dentate gyrus (granule cells); thalamus.	Extrasynaptic (no direct morphological evidence).
α5β3γ2	Less than 5 % of all receptors. High affinity for classical benzodiazepine agonists and antagonist flumazenil. Low affinity for zolpidem.	Hippocampus (pyramidal cells); olfactory bulb (granule cells, periplomerular cells); cerebral cortex; amygdala; hypothalamus; superior colliculus; superior olivary neurons; spinal trigeminal neurons; spinal cord.	Extrasynaptic in hippocampus, cerebral cortex, olfactory bulb. Synaptic and extrasynaptic in spinal trigeminal nucleus and superior olivary nucleus.
α6β2,3γ2	Less than 5 % of all receptors. Insensitive to classical α6β2,3δ benzodiazepine agonists and zolpidem.	Cerebellum (granule cells); dorsal cochlear nucleus.	Synaptic (cerebellar glomeruli) and extrasynaptic on granule cell dendrites and soma.

3.5 Function of $GABA_A$ receptor subtypes

The identification of the pharmacological relevance of $GABA_A$ receptors has been achieved by genetic and medicinal chemistry tools. The genetic approach involved the generation of mouse lines either lacking individual receptor subunits or rendering individual subunits insensitive to the clinically important benzodiazepine site drug diazepam (reviewed by Rudolph and Mohler 2004). The medicinal chemistry approach focused on the development of ligands with selective affinity or efficacy for individual $GABA_A$ receptor subtype (Sternfeld *et al*. 2004, Chambers *et al*. 2004, Atack *et al*. 2005).

3.5.1 α5-$GABA_A$ receptor knock-out mice

The function of $GABA_A$ receptor subtypes has been investigated using knock-out and knock-in mouse lines. Since the present study focuses on α5-$GABA_A$ receptors, the strategies for their investigation will be described in more detail. Knock-out mouse lines for α subunits were generated for α1, α3, α5 and α6 subunit. With the exception of the α3 and α5 subunit knock out mice, all other lines exerted compensatory changes in the expression of other receptor subunits, compromising the interpretation of the phenotype in terms of receptor subtype function (Rudolph and Mohler 2004). In α5 knock out mice, the number of benzodiazepine-binding sites in the hippocampus decreased by 16%, which corresponds roughly to the number of α5-$GABA_A$ receptors in this brain region and suggests no significant up-regulation of other α subunits (Collinson *et al*. 2002). Behaviorally, these mice display an improved performance in the Morris water maze test, a spatial learning task dependent on hippocampal function (Collinson *et al*. 2002). This result was also confirmed with an inverse agonist selective for α5-$GABA_A$ receptors that enhanced performance of rats in the water maze test without any convulsant activity (Chambers *et al*. 2003).

3.5.2 α5-$GABA_A$ receptor knock-in mice

Studies with knock-out mice have shown that elimination of individual α subunits may lead to adaptive compensatory changes during development. To overcome these problems, a point mutation strategy had been developed, which retained the physiological function of the targeted receptor subtype but prevented the modulation of receptor activity by defined drugs. This strategy was perfectly suited for dissecting the

contribution of $GABA_A$ receptors subtypes to the various actions of diazepam, e.g. sedation, muscle relaxation, anticonvulsant activity and anxiolysis. Since α subunits containing a histidine residue in the drug-binding domain (α1H101, α2H101, α3H126, α5H105) convey diazepam-sensitivity to the receptor complex and α subunits with an arginine residue (α4R99, α6R100) diazepam-insensitivity, a histidine to arginine point mutation in α1, α2, α3 or α5 subunits consequently renders the respective $GABA_A$ receptor subtypes insensitive to the actions of diazepam. Generation and analysis of α1(H101R), α2(H101R), α3(H126R) and α5(H105R) knock-in mice revealed first insights in the function of $GABA_A$ receptors subtypes *in vivo* (Rudolph *et al*. 1999, Löw *et al*. 2000, McKernan *et al*. 2000, Crestani *et al*. 2002). The α1-containing $GABA_A$ receptors represent the major subtype (~60% of diazepam-sensitive $GABA_A$ receptor subtypes) and are expressed in virtually all main brain areas (Tab. 1). In α1(H101R) mice the sedative action and the anterograde amnesic action of diazepam was absent, indicating that they are mediated through the α1-$GABA_A$ receptor subtype in the wild type mice (Rudolph *et al*. 1999). The α2 subunit-containing $GABA_A$ receptors represent 15-20% of diazepam-sensitive $GABA_A$ receptors and display a more restricted distribution in brain with particular prominent expression in the limbic system (Tab. 1). Most interestingly, α2(H101R) mice lacked anxiolytic and partially myorelaxant actions of diazepam (Löw *et al*. 2000, Crestani *et al*. 2001). Thus, two major clinically important effects of diazepam are mediated by two distinct $GABA_A$ receptor subtypes: sedation by α1-$GABA_A$ receptors and anxiolysis by α2-$GABA_A$ receptors. In addition, the myorelaxant action of diazepam was reduced in α3(H126R) and α5(H105R) mice, indicating that these receptor subtypes are involved in this effect of diazepam (Löw *et al*. 2000, Crestani *et al*. 2001, 2002).

Immunoblotting, immunohistochamistry and immunofluorescence experiments confirmed the initial expectations that the introduction of the point mutation into the receptor subunits did not change their expressed levels or their regional and subcellular distributions (Rudolph *et al*. 1999, Löw *et al*. 2000). The only exception was the α5(H105R) mouse line, in which the regional and subcellular localization of the α5 subunit remained unaffected but its expression level in the hippocampus was found to be moderately reduced (20%, Crestani *et al*. 2002). Interestingly, the partial deficit of α5-$GABA_A$ receptors in the hippocampus resulted in selective changes in learning and

memory performance. α5(H105R) mice exhibited an improved memory performance in a trace fear conditioning paradigm (Crestani *et al*. 2002) and were resistant to extinction of conditional fear (Yee *et al*. 2004). This indicates that α5-$GABA_A$ receptors, which are mostly expressed extrasynaptically in hippocampus, play a role in the temporal association of threat cues in trace fear conditioning (Crestani *et al*. 2002).

3.6 NMDA receptor signaling cascade underlying learning and memory

Interestingly, a deficit in hippocampal NMDA receptors produces opposite effects on hippocamal learning and memory paradigms than deficits in hippocampal α5-$GABA_A$ receptors. For instance, mice lacking NMDA receptors in the CA1 region of the hippocampus failed to memorize threat cues in trace fear conditioning (Huerta *et al*. 2000), while a deficit in a α5-$GABA_A$ receptors resulted in improved performance in this memory task (Crestani *et al*. 2002). Therefore, both receptors seem to accomplish complementary functions in regulating learning and memory. Since α5-$GABA_A$ receptors are largely located extrasynaptically at spines and shafts of pyramidal cell dendrites, they may directly control and modulate NMDA receptor mediated excitation at the spines.

The hippocampus is critical for converting short-term memories into long-term memories (reviewed by McGaugh 2000). NMDA receptors play an important role in this process as shown by gain- and loss-of-function studies (Tang *et al*. 1999, Shimizu *et al*. 2000). NMDA receptors seem to be required for the induction, but not maintenance of synaptic activity and it is believed that consolidation at a synaptic level is the result of molecular cascades initiated by long-term potentiation triggered during learning (Bourtchuladze *et al*. 1994, Deisseroth *et al*. 1996, Atkins *et al*. 1998, reviewed by Silva *et al*. 1998, Bozon *et al*. 2003, reviewed by Thomas and Huganir 2004). One of the key proteins of the NMDA receptor-induced signaling cascade is the transcription factor CREB (cAMP responsive element binding protein). Genetic and pharmacological studies in rodents demonstrated that CREB is required for a variety of complex forms of memory, including spatial and social learning, indicating that CREB may be a universal modulator of processes underlying memory formation (reviewed by Silva *et al*. 1998).

CREB is a member of a large family of structurally related transcription factors that bind to cAMP responsive element (CRE) sites in certain promoters to enhance gene transcription (reviewed by Lonze and Ginty 2002, Mayr and Montminy 2001).

Transcription factors from the CREB family contain domains that are required for transcriptional activation and domains that mediate dimerization and DNA binding. The activation domain includes a cluster of phosphorylation sites that regulate the activity of CREB. The crucial event in the activation of CREB is the phosphorylation of Ser133 by a variety of kinases and may be a mechanism for the convergence of several different signaling pathways. Thus, CREB activation can be detected in neurons using antibodies specific to phosphorylated Ser133 in the CREB protein (Deisseroth *et al*. 1996). Studies using this approach revealed that while NMDA receptor-dependent synaptic stimulation results in CREB phosphorylation, action potential firing alone does not (Deisseroth *et al*. 1996). This finding indicates that CREB phosphorylation is not a general marker for neuronal activity, but instead responds to specific synaptic signals. Although many kinases can phosphorylate CREB, specific kinases predominate in response to different stimuli. In neurons there is an evidence that the calmodulin kinase pathway is crucial for rapid activity-dependent phosphorylation of Ser133, whereas prolonged activity-dependent phosphorylation is mediated by activation of the Ras-MAPK pathway (Wu *et al*. 2001). Eventually, calcium influx also leads to the dephosphorylation of CREB through activation of protein phosphatases (reviewed by West *et al*. 2002). Hardingham *et al*. (2002) showed that activation of synaptic NMDA receptors leads to the phosphorylation of CREB. In contrast, activation of extrasynaptic NMDA receptors dephosphorylates and inactivates CREB, possibly to prevent overactivation of CREB in case of overspill of synaptic glutamate. These studies indicate that CREB activation and consequently gene transcription is controlled and modulated by NMDA receptors in a complex manner.

4 Aim of the study

α5-$GABA_A$ receptors are most abundantly expressed in the hippocampus, where they mediate tonic inhibition and play an important role in learning and memory. Mice containing the (H105R) point mutation in the α5 subunit gene express reduced levels of the α5 subunit protein in the hippocampus, which resulted in enhanced trace fear conditioning (Crestani *et al*. 2002). Since α5 mRNA levels were not reduced, the mutation seems to influence posttranscriptional events such as synthesis, assembly, trafficking or degradation. To gain insights into how the α5(H105R) mutation affects α5-$GABA_A$ receptors, the present study aimed primarily at analyzing the effect of the mutation on the assembly and trafficking of α5-$GABA_A$ receptors. In addition, we intended to analyze the effect of a reduced expression of α5-$GABA_A$ receptors on the activity of downstream signaling proteins important for learning and memory.

4.1 Analysis of α5-$GABA_A$ receptor targeting in HEK293 and neuronal cells

Our first hypothesis was that the reduced α5 subunits protein levels were caused by an impaired trafficking of α5(H105R)-$GABA_A$ receptors. In order to monitor and compare targeting of α5- and α5(H105R)-$GABA_A$ receptors expressed in HEK293 cells and primary cultured neurons, we used immunocytochemistry and confocal laser scanning microscopy in combination with various tagging methods (antibody, GFP, biarsenical dye- and α-bungarotoxin-based tracking methods).

4.2 Analysis of the α5-$GABA_A$ receptor assembly

The second hypothesis for the reduced α5 subunit protein expression in α5(H105R) mice was that the mutation might be critical for the correct assembly of α5-$GABA_A$ receptors. Therefore we examined whether the H105R mutation might affect the assembly of the receptor, using sucrose density centrifugation analysis and radioligand binding experiments.

4.3 Influence of α5-$GABA_A$ receptors on processes of learning and memory

Previous studies have shown that deletion or reduction of α5-$GABA_A$ receptors enhanced spatial learning (Collinson *et al*. 2002) and temporal learning (Crestani *et al*. 2002) in rodents. As an underlying mechanism, it has been proposed that extrasynaptic

α5-$GABA_A$ receptors modulate the excitatory input arising from NMDA receptors. Therefore, we investigated whether the reduction of α5(H105R)-$GABA_A$ receptors might affect the activity of signaling proteins, which are activated by NMDA receptors and are important for learning and memory.

5 Methods

5.1 Animals

In this study, 8-week old wild type (129/SvJ), α1(H101R) and α5(H105R) mice (> 10 backcrosses to the 129/SvJ background) were used (Rudolph *et al*. 1999, Löw *et al*. 2000, Crestani *et al*. 2002). Rat embryos (embryonic day E18) were obtained from time mated pregnant Wistar rats (RCC, Basel, Switzerland). All experiments were performed in accordance to international guidelines on animal care and use and were approved by the Cantonal Veterinary Office of Zurich.

5.2 Plasmids

The following cDNAs in the appropriate vectors were used in the present study:

cDNA insert	**Vector**	**Source/reference**
$GABA_A$ α1, rat	pBC12/CMV-α1	Benson *et al*. 1998
$GABA_A$ α1 (TetraCys), rat	pβAct-α1TetraCys	This study
$GABA_A$ α1 (TetraCys, myc), rat	pβAct-α1TetraCys-myc	This study
$GABA_A$ α1 (TetraCys, flag), rat	pβAct-α1TetraCys-flag	This study
$GABA_A$ α5, rat	pBC12/CMV-α5	Benson *et al*. 1998
$GABA_A$ α5, rat	pSP72-α5	Benson *et al*. 1998
$GABA_A$ α5, rat	pcDNA3.1-α5	This study
$GABA_A$ α5 (btx), rat	pcDNA3.1-α5-btx	This study
$GABA_A$ α5 (GFP), rat	pCMV-α5-GFP	Gift of Dr. A. Zeller
$GABA_A$ α5(H105R)(GFP), rat	pCMV-α5(H105R)-GFP	This study
$GABA_A$ β2, rat	pBC12/CMV-β2	Benson *et al*. 1998
$GABA_A$ β3, rat	pBC12/CMV-β3	Benson *et al*. 1998
$GABA_A$ γ2-long, rat	pBC12/CMV-γ2	Benson *et al*. 1998

5.4 Cloning

Cloning of the TetraCys tag into the α1 subunit

The complementary oligonucleotides coding for the TetraCys tag (amino acid sequence CCPGCC, Adams *et al*. 2002) were synthesized (Microsynth) with nucleotide sequences GTGACCCCTGTTGTCCTGGCTGTTGCG (sense) and GTCACCG-CAACAGCCAGGACAACAGGG (antisense). Both oligonucleotides had 5' phosphate groups and were annealed to each other by heating to 95°C and slowly cooling down. The insert was subcloned into the BstEII site in the second intracellular loop of the α1 subunit (amino acid 383) using the Ready-to-go ligation kit (Amersham). The α1TetraCys construct was then subcloned into the pβAct vector (containing the chicken-β-actin promoter) via HindIII and XbaI sites using the LigaFast rapid ligation kit (Promega). Plasmids were transformed into *E. coli* (XL-10 gold, Stratagene) and were isolated with the Nucleobond kit (Macherey-Nagel, Germany) according to manufacturer's protocol.

Cloning of myc and flag tags into the α1 subunit

The myc or flag tag was introduced at the C-terminus of the α1TetraCys construct in the pβAct vector. The tags were appended using the forward primer CCCGTGAAGCT-TATGAAGAAAAGTCGGGGTCTC and either the reverse primer AACTCTAGAC-TACAGGTCCTCCTCTGAGATCAGCTTCTGCTCCTCTTGATGGGGTGTGGG for the myc tag or: AACTCTAGACTACTTGTCGTCGTCGTCCTTGTAGTCTTGATG-GGGTGTGG for the flag tag. The new constructs were amplified by PCR with 50 ng template α1TetraCys-pβAct, 125 ng of forward and reverse primer, 0.2 mM dNTPs, 1.5 mM Mg^{2+}, 5 units Taq polymerase and reaction buffer (Invitrogen). The PCR was performed at a melting temperature of 55°C for 30 cycles (Mastercycler, Eppendorf). The α1TetraCys-myc and α1TetraCys-flag inserts were cut with HindIII and XbaI restriction enzymes and were subcloned via these sites into the pβAct vector using the LigaFast rapid ligation kit (Promega). Plasmids were transformed into *E. coli* (XL-10 gold, Stratagene) and were isolated with the Nucleobond kit (Macherey-Nagel, Germany).

Cloning of the bungarotoxin (btx) tag into the α5 subunit

The complementary oligonucleotides coding for minimal α-bungarotoxin binding site (amino acids WRYYESSLEPYPD, Sekine-Aizawa and Huganir 2004) were synthesized (Microsynth, sense: CTAGCTGGAGATACTACGAGAGCTCCCTGG-AGCCCTAC-CCTGACA, antisense: CTAGTGTCAGGGTAGGGCTCCAGGGAGC-TCTCGTAGTATCTCCAT). The two fragments contained 5' phosphate group and were annealed to each other by heating to 95°C and slowly cooling down. The insert was subcloned into the SpeI site of the α5 subunit (amino acid 36) in the pSP72 cloning vector using the LigaFast rapid ligation kit (Promega). The α5btx construct was finally subcloned into the pcDNA3.1 vector (Invitrogen) via KpnI and BamHI sites (LigaFast rapid ligation kit, Promega). Plasmids were transformed into *E. coli* (XL-10 gold, Stratagene) and were isolated with the Nucleobond kit (Macherey-Nagel, Germany).

Cloning of the α5(H105R)-GFP construct

The GFP-tagged α5 subunit in the pBC12/CMV vector was a kind gift from Dr. A. Zeller. To generate the pBC12/CMV-α5(H105R)-GFP construct, the His at position 105 was exchanged by Arg using site-directed mutagenesis. Oligonucleotides designed for this purpose contain the desired mutation (CAC into CGG) and anneal to the same sequence on opposite strands of the plasmid. The oligonucleotide sequences were as following: CCAGACACGTTCTTCCGGAACGGCAAGAAGTCC (sense) and GGACTTCTTGCCGTTCCGGAAGAACGTGTCTGG (antisense). Quick-change site-directed mutagenesis was performed using PCR. The PCR mix contained 50 ng pBC12/CMV-α5-GFP template, 125 ng of each oligonucleotide, 0.2 mM dNTPs, 1 mM Mg^{2+}, 2.5 units Pfu polymerase and reaction buffer (Promega). The PCR was performed at a melting temperature of 55°C for 16 cycles (Mastercycler, Eppendorf). The template DNA was digested using DpnI (BioLabs). The new plasmid carrying the point mutation was transformed into DH5α *E. coli* cells (Stratagene) and isolated with the Nucleobond kit (Macherey-Nagel, Germany).

5.5 Cell culture and transfection

5.5.1 Culture and transfection of HEK293 cells

Human embryonic kidney (HEK293) cells were cultured in minimal essential medium (MEM, Invitrogen) containing 10% fetal calf serum (Invitrogen). Transfection of HEK293 cells was performed using the calcium phosphate precipitation method according to Chen and Okayama (1987). The transfection solution for a 10 cm culture dish (Falcon) was prepared by filling up the appropriate DNA amount with water to a volume of 720 µl and supplemented with 80 µl 2.5 M $CaCl_2$ and 800 µl BES buffer (50 mM BES pH 6.9, 280 mM NaCl, 1.5 mM Na_2HPO_4). The mixture was incubated for 15 min at room temperature and added dropwise to the plated cells. In a 3 cm culture dish the amount of transfection solution was scaled down 10-fold. After addition of the transfection solution, the cells were incubated overnight at 35°C and 3% CO_2. The next day, the cells were washed with PBS and maintained in the fresh culture medium for 48 h at 37°C and 5% CO_2. For immunofluorescence staining, HEK293 cells were plated at a density of 0.5 x 10^6 cells per 3 cm culture dish and transfected with a total amount of 4 µg of DNA per dish.

5.5.2 Culture of cortical neurons

Primary cultures of cortical neurons were prepared from rat embryos taken at embryonic day E18 from pregnant wistar rats. The cortex was dissected on ice in PBG buffer (PBS pH 7.4, 10 mM glucose) and incubated for 15 min at 37°C in papain solution (PBS pH 7.4, 10 mM glucose, 1 mg/ml bovine serum albumin, 10 ug/ml DNAse-I, 0.5 mg/ml papain). Subsequently neurons were dissociated by gentle trituration with a fire-polished Pasteur pipette and suspended in DMEM (Invitrogen) containing 10 % fetal calf serum (Invitrogen). Neurons were plated to a density of 40 000 to 60 000 onto poly-L-lysine coated (100 µg/ml) glass coverslips (∅ 15 mm, 0.17 ± 0.02 mm thickness, Karl Hecht Glaswarenfabrik, Germany). The neurons were cultured either in the absence or in presence of a glial feeder layer.

Without glial feeder layer: after one hour, neurons were attached to the coverslips and transferred to a 12-well plate in the same medium (1 ml). After 24 hours, the medum was replaced with NB medium (Neurobasal medium (Invitrogen) containing B27 supplement 1:50 (Invitrogen), GlutaMAX 1:100 (Invitrogen), antimycoticum /antbioticum 1:100 (Invitrogen), FUDR 1:1000 (fluorodeoxyuridine 10 mM and uridine

10 mM, Sigma; to stop glial cell proliferation). Cultures were kept at 37°C and 5 % CO_2 in a humidified incubator.

With glial feeder layer: glial cells, prepared from rat cortex of postnatal day 0 (P0), were plated in advance in 12-well plates and cultured in DMEM containing 10 % fetal calf serum to generate a feeder layer. After preparation of cortical neurons, the glial cell culture medium was exchanged with a defined serum-free medium (Brewer and Cotman, 1989) and neurons, after attaching to coverslips for one hour, were placed above the feeder layer. Cultures were kept at 37°C and 10 % CO_2 in a humidified incubator.

5.5.3 Transfection of primary cortical neurons

Nucleofection

Freshly dissociated cortical neurons were transfected by means of the Amaxa nucleofection technology (Amaxa Biosystems, Germany) according to manufacturer's protocol using 2 μg DNA and electroporation program O-03. After nucleofection, neurons were plated at a density of 30-40 000 per cover slip (∅ 18 mm) and kept under the same conditions as nontransfected cells.

NeuroFect

Cortical neurons were grown for 4-6 days and transfected with the NeuroFect transfection agent (Genlantis, San Diego, CA). In separate tubes, 1 μg of NeuroFect diluted in 50 μl serum free medium (OptiMEM, Invitrogen) and 1 μg DNA in serum free medium were prepared. The diluted NeuroFect was added dropwise to the DNA solution followed by gentle mixing. The NeuroFect/DNA solution was incubated for 15-20 min at room temperature to allow the formation of polyplexes. Old culture medium was removed from neurons and replaced with 400 μl fresh medium and 100 μl NeuroFect/DNA solution was added on top. Transfected neurons were incubated at 37°C and 5 % CO_2 and gene expression was assayed 24-48 h post transfection.

5.6 Immunocytochemistry

5.6.1 Conventional double and triple labeling immunocytochemistry and confocal microscopy

Double and triple labeling immunocytochemistry was performed on HEK293 cells and primary cortical neurons. Cells were fixed for 10 min with 4% paraformaldehyd and 4 % sucrose in 150 mM phosphate buffer pH 6.8-7.4 (PFA) and washed three times in phosphate buffered saline pH 7.4 (PBS). For labeling of both surface and intracellular proteins, cells were permeabilized for 5 min with 0.5% Triton X-100 in PBS and washed once with PBS. Labeling was achieved by incubating cells with a primary antibody diluted in PBS containing 10% normal goat serum (NGS). After washing three times for 10 min in PBS, cells were incubated with secondary antibody coupled to a green (Alexa green 488, Molecular Probes, 1:1000), red (Cy3, Jackson ImmunoResearch, 1:500) and/or blue (Cy5, Jackson ImmunoResearch, 1:100) fluorescent fluorophore. Cells were washed again three times in PBS to remove unbound antibodies and mounted on glass slides with mounting medium (DAKO Cytomation, Carpinteria). Specimens were analyzed with a confocal laser scanning microscope (Axioplan2, LSM 510 Meta, Carl Zeiss AG, Germany) equipped with a 100x oil immersion objective (N.A. 1.4). Images were acquired by sequential scanning of the emission lines and using the full dynamic range of the photodetectors. Digital images were processed using the software Imaris, version 4.1 (Bitplane, Switzerland). For display, minimal linear contrast adjustments were made.

5.6.2 Biarsenical dye labeling

Prior to labeling, HEK293 cells or primary cortical neurons expressing the α1TetraCys construct were washed once with PBS buffer. Lumio green and Limio red dyes (Invitrogen) were diluted in PBS buffer containing 1 mM pyruvic acid, 10 μM 1,2-ethanedithiol (EDT_2, Fluka), and added to the cells to a final concentration of 1 μM (Lumio green) and 2.5 μM (Lumio red). Incubation proceeded for 45 min at 37°C or at room temperature in a humid chamber. To remove unbound Lumio dyes, cells were washed five times for 5 min with PBS containing 250 μM EDT_2. Cells were then fixed with PFA and processed for further antibody staining as described above.

5.6.3 Alexa-Fluor488-α-bungarotoxin labeling

Living HEK293 cells or cortical neurons transfected with α5btx constructs were labeled with 1 μg/ml Alexa-Flour488-conjugated α-bungarotoxin (Invitrogen) diluted directly into the cell culture medium. Cells were incubated for 30 min in a humid chamber at 37°C and 5 % CO_2. After labeling, the cells were washed three times with prewarmed buffer (2 mM $CaCl_2$, 2 mM $MgCl_2$, 1 uM glycine, 5 mM KCl, 119 mM NaCl, 25 mM HEPES, 30 mM glucose, pH 7.4). Subsequently, the cells were fixed with PFA and further processed for antibody staining as described above.

Labeling of transfected cortical neurons additionally included a preincubation step with tubocurarine (10 μg/ml) for 15 min at 37°C and 5 % CO_2 to block endogenous α-bungarotoxin binding sites of α7 nicotinic acetylcholine receptors.

5.7 Cell surface enzyme-linked immunosorbent assay (ELISA)

HEK293 cells grown on 10 cm culture dishes were transiently transfected with the subunit combinations α5β3γ2 or α5(H105R)β3γ2 using a total of 20 μg DNA and splitted after 24 h onto 96-well plates (Nunc). After further 48 h, cells were fixed with PFA. For cell surface staining of α5-$GABA_A$ receptors, cells were washed in PBS and incubated with α5 antibody (1:2000) diluted in PBS/10% NGS for 1 hour at room temperature. Cells were then washed three times for 10 min with PBS and incubated with horseradish-peroxidase conjugated anti-guinea pig antibody (1:1000) for 1 hour at room temperature. Cells were again extensively washed and the activity of horseradish-peroxidase was detected using tetramethylbenzidine as a substrate (100 μl/well; 0.24 mg/ml tetramethylbenzidine, 0.2 M sodium citrate pH 3.95, 0.03 % H_2O_2). The colorimetric reaction was terminated after 20-30 min by adding 100 μl 2 M H_2SO_4 and the optical density was recorded at 450 nm in a microplate reader (Synergy HT, Biotek). Total expression of α5 subunit was determined in cells that were permeabilized with 0.5 % Triton X-100 for 5 min at room temperature after fixation with PFA. Nonspecific antibody reaction was determined in parallel cultures of non-transfected cells.

5.8 Membrane preparation

Brains from 8-week-old mice were homogenized in 10 volumes of 10 mM Tris-HCl, pH 7.4, 0.32 M sucrose, 5 mM EDTA, 0.02 % NaN_3, protease inhibitors (complete mini, Roche), 0.1 mM phenylmethylsulphonyl fluoride and centrifuged at 1000 x g for 10

5.3 Antibodies

The following antibodies were used in this study:

Name	**Type**	Dilution	**Source/Reference**
α1	pR Ab	1:5000 IF	Fritschy & Mohler 1995
α1C	pR Ab	1:20 IP	Benke *et al*. 1991
α2C	pR Ab	1:20 IP	Marksitzer *et al*. 1993
α3N	pR Ab	1:15 IP	Benke *et al*. 1991
α5	pGP Ab	1:3000 IF	Fritschy & Mohler 1995
		1:2000 WB	
		1:2000 ELISA	
β2/3 (clone BD17)	mM Ab	1:4000 IF	Schoch *et al*. 1985
		1:2000 WB	
β-actin	mM Ab	1:10000 WB	Chemicon
CaMKII	pR Ab	1:1000 WB	Cell Signaling
CREB	pR Ab	1:1000 WB	Cell Signaling
Flag	mM Ab	1:2000 IF	Sigma-Aldrich
γ2	pGP Ab	1:2000 IF	Fritschy & Mohler 1995
		1:2000 WB	
GFP	mM Ab	1:1000 IF	Clontech
Myc (clone 9E10)	mM Ab	1:4000 IF	Sigma-Aldrich
p44/42 MAPK	pR Ab	1:1000 WB	Cell Signaling
phospho-CaMKII(Thr 286)	pR Ab	1:1000 WB	Cell Signaling
phospho-CREB(Ser133)	pR Ab	1:1000 WB	Cell Signaling
phospho-38MAPK(Thr180/Tyr182)	pR Ab	1:1000 WB	Cell Signaling
phospho-PKCδ(Thr 505)	pR Ab	1:1000 WB	Cell Signaling
phospho-PKCε(Ser 719)	pR Ab	1:1000 WB	Cell Signaling
phospho-SAPK/JNK(Thr183/Tyr185)	pR Ab	1:1000 WB	Cell Signaling
phospho-Src(Tyr 416)	pR Ab	1:1000 WB	Cell Signaling

Ab, antibody; m, monoclonal; p, polyclonal; M, mouse; R, rabbit; GP, guinea pig; IF, immunofluorescence; IP, immunoprecipitation; WB, Western blot.

min. The pellet was washed once and the combined supernatants were centrifuged for 20 min at 12 000 x g. The resulting crude membrane pellet was resuspended in buffer and the protein content was determined using either the Bradford (BioRad) or the BCA (Pierce) protein assay according to supplier's instructions. For some radioligand binding experiments the homogenate was directly centrifuged for 20 min at 12 000 x g. The crude membranes were stored at -80°C until used.

5.9 Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) and Western blotting

Crude membranes or homogenates were thawed, supplemented with SDS-sample buffer (62.5 mM Tris pH 6.8, 10 % glycerol, 2.3 % SDS, 0.025 % bromphenolblue, 5 % 2-mercaptoethanol) and heated for 5 min at 95°C. Samples were separated by SDS-PAGE according to Lämmli (1970). Following electrophoresis in 10 % mini gels (Mini Protean II, BioRad), proteins were transferred onto a nitrocellulose membrane (0.45 um, EGT Chemie) using a wet blotting device (BioRad) and 15 mM phosphate pH 6.8-7.5, 0.05 % SDS and 20 % MeOH as transfer buffer. The transfer was performed at room temperature for one hour at 250 mA. The blotting efficiency was checked by reversible staining of proteins with 0.1 % amidoblack solution. Remaining protein binding sites on the nitrocellulose membrane were blocked with TBST containing 5 % non-fat milk powder (blocking solution) for at least one hour at room temperature. For immunodetection, the blots were incubated overnight at 4°C with the respective primary antibody diluted in blocking solution. The blots were washed once for 10 min with RIPEA (20 mM Tris pH 7.5, 60 mM NaCl, 2 mM EDTA, 0.4 % SDS, 0.4 % Triton X-100, 0.4 % deoxycholate) and three times for 15 min with TBST (20 mM Tris pH 7.5, 137 mM NaCl, 0.05 % Tween). Subsequently, the blots were incubated for one hour with the appropriate HRP-conjugated secondary antibody (1:5000, Jackson Immunoresearch) diluted in blocking solution. After washing the blots with RIPEA for 10 min and three times for 15 min with TBST, immunoreactivity was detected by the enhanced chemoluminescence method (Pierce, Supersignal West Pico Chemiluminiscent Substrate) according to the supplier's recommendations. Chemolumiscence was captured using a LAS-1000plus image analyzer (Fujifilm) and quantification of immunoreactive bands was performed with the AIDA software (version 3.25, Raytest, Germany). To ensure an analysis in the linear range, samples of

increasing protein concentrations were applied to SDS-PAGE and the blots were exposed for various times.

5.10 Sucrose density gradient centrifugation

For solubilization of $GABA_A$ receptors, crude membranes were thawed and washed once in 10 mM Tris pH 8, 150 mM NaCl, 1 mM EDTA, protease inhibitors (complete mini), and resuspended in the same buffer to give a protein concentration of 5 mg/ml. Following addition of sodium deoxycholate to a final concentration of 0.5 %, the mixture was incubated for 30 min on ice. Insoluble material was removed by centrifugation for 30 min at 100,000 x g. The supernatant was carefully removed and subjected to sucrose density centrifugation analysis. Linear sucrose gradients were prepared from 5 and 20 % sucrose in 50 mM Tris pH 8, 0.02 % NaN_3, 0.5 % Triton X-100. Deoxycholate extracts (600 µl) were layered on the top of each gradient (10 ml) and were centrifuged at 170 000 x g for 15 h at 4°C. After centrifugation, fractions of 350 µl were collected and analyzed for α5, β2/3 or γ2 immunoreactivity by Western blotting as well as for [^{3}H]Ro15-4513 and [^{3}H]L655,708 binding (see below). Molecular size calibration was achieved using three marker proteins (4 mg/ml bovine serum albumine, aldolase and catalase) on gradients run in parallel. The presence of marker proteins in each fraction was determined by SDS-PAGE and staining with Coomassie brilliant-blue.

5.11 Radioligand binding assays

5.11.1 Receptor autoradiography

The regional distribution of [^{3}H]L655,708 and [^{3}H]Ro15-4513 binding was analyzed on unfixed parasagittal cryostat-cut sections (12 µm) of adult wild type and α5(H105R) knock-in mouse brains. Slide-mounted sections were thawed and preincubated in 100 mM Tris-HCl pH 7.4 for 15 min at 4°C, followed by incubation for 90 min with 20 nM of [^{3}H]Ro15-4513 or 2 nM [^{3}H]L655,708. Sections were washed three times for 15 s in buffer and finally dipped in distilled water. Subsequently, sections were dried and exposed to a tritium-sensitive phosphor screen (Packard Cyclone Storage Phosphor System, Packard, Meridian, CT) for 3-7 days. Nonspecific [^{3}H]Ro15-4513 as well as [^{3}H]L655,708 binding was assessed in parallel assays including 10 µM flumazenil. The

screens were scanned with a Packard Cyclone Scanner and labeling intensities were quantified using tritium standards (Microscale, Amersham) exposed in parallel.

5.11.2 Saturation binding experiments

For saturation binding experiments, crude mouse brain membranes were thawed and washed once with 50 mM Tris-HCl pH 7.4. Aliquots containing 100 μg protein were incubated with increasing concentrations (0.25, 0.5, 1, 1.5, 2, 3, 5, 7.5, 10, 12.5, 15, 20 nM) of [^{3}H]L655,708 (83 Ci/mmol, Amersham Biosciences) in a total volume of 0.2 ml for 90 min on ice. Nonspecific [^{3}H]L655,708 binding was assessed at each radioligand concentration by inclusion of 10 μM flumazenil in the reaction. The incubation was stopped by rapid vacuum filtration on Whatman GF/C filters using a semiautomatic cell harvester (Skatron Instruments). Dried filters were subjected to liquid scintillation counting using 4 ml Ultima Gold as scintillation fluid. The binding data were analyzed using the program 'KELL for Windows 6.0.5' (Biosoft, UK).

5.11.3 Competition binding assay

For competition binding assays, mouse forebrain was homogenized and centrifuged at 12000 x g. Aliquots of the resuspended pellet (300 μg protein/vial) were incubated in triplicates with 6 nM of [^{3}H]Ro15-4513 and increasing concentrations of zolpidem (0, 0.5, 1, 2.5, 5, 10, 25, 50, 100, 250, 500, 1 000, 2 500, 5 000, 10 000, 25 000, 50 000, 100 000 nM). After 90 min of incubation on ice, the reaction mixtures were vacuum-filtered through GF/C Whatman filters using a semiautomatic cell harvester (Skatron Instruments) and subjected to liquid scintillation counting. Nonspecific binding was determined in the presence of excess flumazenil (10 μM). Ligand binding data were analyzed using the program 'KELL for Windows 6.0.5' (Biosoft, UK).

5.11.4 Ligand binding to immunoprecipitated $GABA_A$ receptors

Membranes prepared from forebrains of wild type or α5(H105R) mice, were solubilized using 0.5 % deoxycholate (see above) and aliquots (1 ml) were incubated with 20 μl α1C, 20 μl α2C or 30 μl α3N antiserum, overnight at 4°C. Subsequently, 200 μl Pansorbin (suspension of 10 % Staphylococcus aureus; Calbiochem) was added and the mixture incubated 90 min at 4°C with constant rotation. After four times washing with solubilization buffer (10 mM Tris/HCl pH 8, 150 mM NaCl, protease inhibitors

(complete mini), 0.5 % deoxycholate) the precipitates were resuspended in the same buffer and probed in triplicates for [^{3}H]Ro15-4513 (12 nM) and [^{3}H]L655,708 (2 nM) binding (total reaction volume of 0.2 ml). After 90 min at 4°C, the samples were filtered through Whatman GF/C filters, which were pretreated with 0.1 % polyethyleneamine for 30 min at room temperature. Filtration was done using a manual filtration device (Milipore). The filters were rapidly washed three times with 4 ml ice-cold assay buffer (10 mM Tris-HCl pH 7.4, 150 mM NaCl) and subjected to scintillation counting. Nonspecific radioligand binding was determined by including 10 μM clonazepam in parallel incubations.

6 Results

$GABA_A$ receptors containing the α1, α2, α3 or α5 subunit mediate fast inhibitory neurotransmission that can be enhanced by allosteric modulation via the benzodiazepine binding site. Since they bind the benzodiazepine site agonist diazepam, these receptor subtypes are classified as diazepam-sensitive $GABA_A$ receptors. In contrast, receptors containing either the α4 or α6 subunit do not bind benzodiazepine site full agonists and thus constitute the class of diazepam-insensitive $GABA_A$ receptors. Diazepam-sensitivity critically depends on a conserved histidine residue in the extracellular located N-terminal domain of the α subunits. This histidine residue is involved in formation of the benzodiazepam binding site at position 101 in α1 and in α2, 126 in α3 and 105 in α5 subunit. Mutation of the histidine residue to an arginine renders the respective receptor insensitive to diazepam binding. This point mutation was used to dissect the contribution of receptor subtypes containing distinct α subunits to the diverse actions of diazepam by generating α1(H101R), α2(H101R), α3(H126R) and α5(H105R) knock-in mouse lines (Rudolph *et al*. 1999, Löw *et al*. 2000, Crestani *et al*. 2002). In α5(H105R) mice, most actions of diazepam were similar to the wild type mice. However, α5(H105R) mice displayed changes in learning and memory performance caused by a selective reduction of α5 subunit protein in the hippocampus (Crestani *et al*. 2002). Since α5 mRNA levels were not reduced, the mutation seemed to influence posttranscriptional events in α5 subunit synthesis/degradation and/or receptor assembly. Our hypothesis was that the point mutation plays specific role in α5-$GABA_A$ receptor assembly or trafficking, issues that we aimed to analyze in HEK293 cells, cultured neurons and mouse brain tissue.

The major experimental limitation we had to deal with was the lack of sufficient amounts of high-quality α5 subunit-selective antibodies. The high-quality antiserum available works exceptionally well in the immunocytochemical staining methods but would have been needed to be affinity-purified to work equally well for Western blotting. Since this antibody was raised in Guinea pigs, which results in only very small amounts of serum, affinity-purification of this precious antibody was prohibited. All attempts to generate a new suitable α5 subunit-selective antiserum failed so far. We tested dozens of bleedings from immunized Guinea pigs and rabbits for its suitability for Western blotting and immunoprecipitation and were not able to identify a single one.

In addition, the only commercial available α5 subunit-selective antibody (Abcam) yielded only very weak staining in Western blots and was therefore useless.

These experimental constraints prevented a detailed Western blot analysis of the expression levels of the α5 subunit in different brain areas and on the subcellular level. All our attempts failed due to the limited sensitivity of the available antiserum in this assay. More importantly, we had no α5-selective antiserum for immunoprecipitation studies at our disposal, which prevented the direct isolation and analysis of the receptor population containing the α5 subunit.

6.1 Expression level of α5 subunits in wild type and α5(H105R) mouse brain

α5-$GABA_A$ receptors display a particularly restricted distribution being most abundant in the hippocampus where they account for 22 ± 2 % of $GABA_A$ receptors (Sur *et al*. 1998). Introduction of the H105R point mutation in the α5 subunit gene caused a reduction of α5 subunit protein expression by 20 ± 5 % in whole mouse brain (Crestani *et al*. 2002). As a first step in the biochemical analysis of α5(H105R)-$GABA_A$ receptors we determined the expression levels of α5 subunits in cortex/hippocampus preparations of α5(H105R) knock-in mice. Cortex/hippocampus tissue derived from 15 wild type and 15 α5(H105R) knock-in mice were divided into three pools of five animals each and were analyzed by Western blotting for α5 subunit expression. To ensure linearity of the signal, α5 subunit protein levels were measured at different protein concentrations and chemiluminiscence was captured using a highly light sensitive CCD camera (Fuji LAS 1000 imaging system) that enables measurements within a very broad dynamic range. At all protein concentrations tested, α5 subunit staining was considerably weaker in the hippocampus/cortex preparations of α5(H105R) knock-in mice than in wild type mice (Fig. 1A). Quantification of the Western blots revealed a 23 ± 2 % reduction of α5(H105R) expression (Fig. 1B). This result fits very well to the data published on whole mouse brain (20 ± 5 %, Crestani *et al*. 2002).

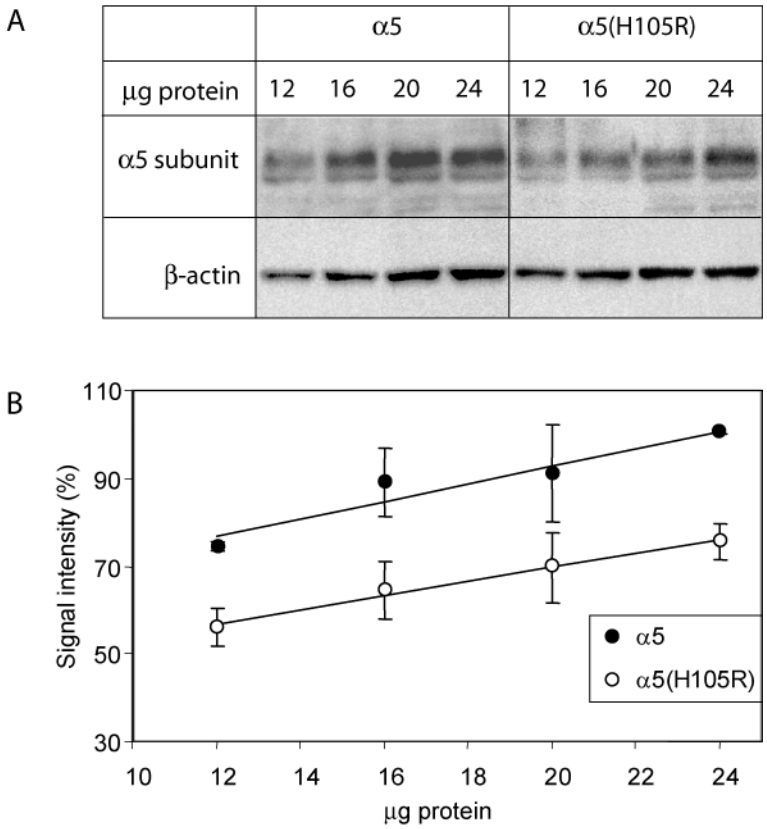

Figure 1. Expression level of α5 subunit in the forebrain of wild type and α5(H105R) knock-in mice. (A) Western blot of cortex/hippocampus preparations from wild type and α5(H105R) knock-in mice showed a reduction of the α5 subunit level at all protein concentration tested. β-actin levels were tested in parallel and served as controls for loading of equal amounts of protein. (B) Quantification of Western blot signals revealed a 23 ± 2 % reduction of α5(H105R) subunit expression. The signal intensity at 24 μg protein in wild type mice was set to 100%. The experiment was done three times using three pools of mice for each genotype (5 animals per pool).

6.2 Expression and cell surface targeting of α5- and α5(H105R)-GABA$_A$ receptors in HEK293 cells

To investigate potential causes for the observed reduction of α5(H105R)-GABA$_A$ receptors, we hypothesized that the α5(H105R) mutation might affect targeting of the receptor complex to the cell surface. This hypothesis was tested using GABA$_A$ receptors expressed in HEK293 cells, where defined subunit combinations can be expressed and their subcellular localization easily monitored.

To analyze whether the α5(H105R) mutation affects cell surface expression of the receptor complex, HEK293 cells were transiently transfected with plasmids coding for β3, γ2 and either α5 or α5(H105R) subunits. The cells expressing α5- or α5(H105R)-$GABA_A$ receptors were fixed and permeabilized with Triton X-100 to label all α5 subunits irrespective of their subcellular localization (Fig. 2, left panel). Alternatively, cells were not permeabilized to selectively detect α5 receptors on the cell surface (Fig. 2, right panel). Both α5 and α5(H105R) subunits were abundantly found in intracellular compartments and at the plasma membrane. Visually inspected, the signal intensities for the wild type α5 subunit and the α5(H105R) subunit appeared similar, suggesting comparable levels of receptor expression within the cells and at the cell surface.

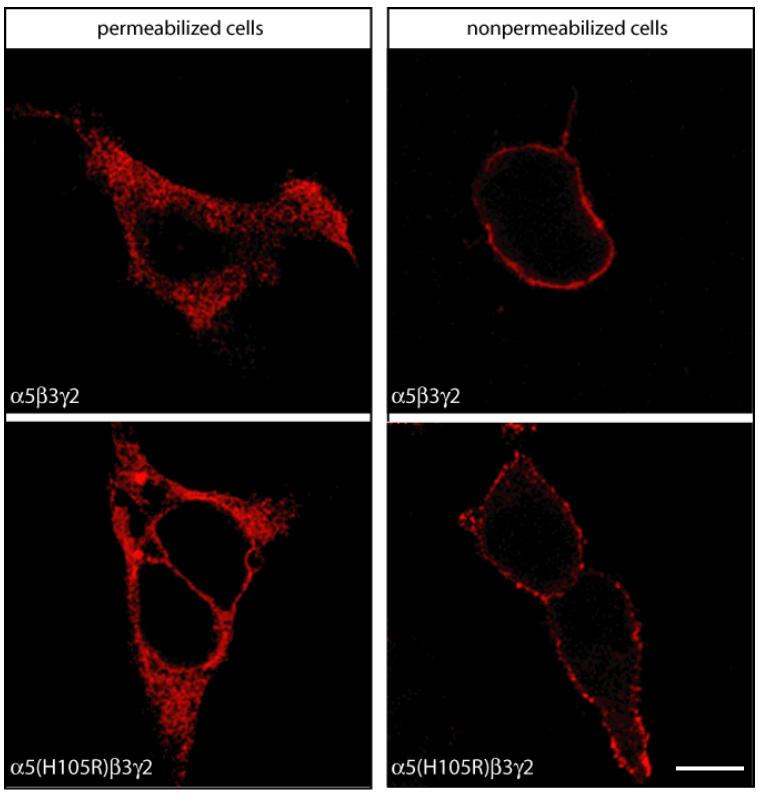

Figure 2. Immunofluorescence staining of the α5 subunit in HEK293 cells transiently transfected with the α5β3γ2 or α5(H105R)β3γ2 subunit combination. The cDNAs of α5 or α5(H105R) subunits combined with β3 and γ2 cDNAs were introduced into HEK293 cells via calcium phosphate transfection. After 48 hours of expression, cells were immunostained using α5 subunit-selective antibodies and visualized by confocal scanning microscopy. Cells permeabilized with Triton X-100 resulted in labeling of total α5 protein content (left panel). Staining of nonpermebilized cells (right panel) retained an intact plasma membrane and resulted in detection of cell surface receptors. Total expression as well as cell surface expression of α5 and α5(H105R) receptors appeared similar. Scale bar: 10 μm.

In order to quantify total and cell surface expression of the receptors, HEK293 cells expressing the α5β3γ2 and α5(H105R)β3γ2 subunit combinations were subjected to enzyme-linked immunosorbent assay (ELISA). Transfected cells grown on 96-well plates were probed with α5 subunit-specific antibodies either in non-permeabilized cells for detection of cell surface receptors or in cells permeabilized with Triton X-100 for detection of total expression of receptors (cell surface and intracellular receptors). Bound α5 antibodies were detected via a colorimetric reaction and quantified using a microplate reader. The measurements revealed comparable signals for both α5 and α5(H105R) subunits with optical densities of 0.206 ± 0.071 and 0.204 ± 0.023, respectively (Tab. 1). This finding indicates similar transfection and expression levels of α5 and α5(H105R) subunits in HEK293 cells. The plasma membrane inserted α5 and α5(H105R) receptors were calculated as a percentage of total α5 and α5(H105R) subunits expressed and amounted to 60 ± 4 % for α5 receptors and 56 ± 5 % for α5(H105R) receptors in HEK293 cells (Tab. 1).

These experiments indicate that - in contrast to neurons *in situ* - expression and cell surface targeting of α5(H105R) receptors appeared not to be affected in this cell type.

Table 1. ELISA revealed similar plasma membrane insertion of both α5β3γ2 and α5(H105R)β3γ2 receptors in HEK293 cells.

subunit combination expressed in HEK293 cells	receptor surface expression (% of total α subunit expressed)	optical density (α subunit expression)
α5β3γ2	60 ± 4	0.206 ± 0.071
α5 (H105R) β3γ2	56 ± 5	0.204 ± 0.023

HEK293 cells expressing the α5β3γ2 or α5(H105R)β3γ2 subunit combinations were grown on 96-well plates and subjected to ELISA. Cells were fixed with PFA and either nonpermeabilized for detection of cell surface receptors or permeabilized with Triton X-100 for determination of total expression of receptors (cell surface and intracellular receptors). After labeling with α5 subunit-specific antibodies, cells were incubated with HRP-conjugated secondary antibodies. HRP activity was detected via a colorimetric reaction using TMB as substrate and quantified using a microplate reader. Nonspecific staining was determined in parallel cultures of nontransfected cells. The results are given as mean ± SD from four independent experiments.

6.3 Targeting of α1β2γ2 and α5β3γ2 $GABA_A$ receptors in cultured neurons

The expression levels and targeting to the cell surface of α5- and α5(H105R)-$GABA_A$ receptors in HEK293 cells were found to be essentially indistinguishable and suggests that the assembly and trafficking of receptor complexes in these cells is unaffected by the point mutation. This finding is in striking contrast to the reduced expression levels of α5(H105R)-$GABA_A$ receptors observed in brain. This apparent discrepancy might be due to overexpression of α5-receptors in HEK293 cells, which may override subtle effects of the point mutation on either targeting efficiency and/or turnover of α5(H105R)-$GABA_A$ receptors. Therefore, we aimed at analyzing trafficking of α5 receptors within neurons using novel tagging techniques for receptor visualization and tracking in living cells. Both, the biarsenical-tetracysteine labeling and α-bungarotoxin labeling techniques have been reported to be applicable on living neurons (Ju *et al.* 2004, Sekine-Aizawa and Huganir 2004) and were expected to be powerful tools to investigate α5 and α5(H105R) receptors trafficking.

6.4 Biarsenical-tetracysteine labeling of α1TetraCysβ2γ2 receptors in HEK293 cells and cultured neurons

To investigate the trafficking of α5-$GABA_A$ receptors, we first aimed at establishing the non-invasive biarsenical-tetracysteine labeling method (Zhang *et al.* 2002, Ju *et al.* 2004). This method allows the site-specific labeling of a protein upon introduction of a small tetracysteine tag CCPGCC, which is recognized by membrane-permeable red (Lumio red) or green (Lumio green) fluorescent biarsenical dyes (Zhang *et al.* 2002). These organic dyes are designed to become fluorescent upon covalent binding to the sulphur atoms of the cysteines in the tag. The most important feature is that this kind of labeling can be conducted in living cells where the tagged protein can be visualized and tracked during a time course of hours. In addition, this method permits pulse-chase experiments, which are required for the intended study on the trafficking of $GABA_A$ receptors.

As a model system to test the labeling method, we used the α1-$GABA_A$ receptor subtype transiently expressed in HEK293 cells. Since the binding of biarsenical flourophores requires reducing conditions present in the cytoplasm, the tetracysteine tag (CCPGCC) was cloned into the large intracellular loop of the α1 subunit (amino acid

383) and subsequently the specificity of Lumio red and Lumio green dyes for the inserted tag was tested. To avoid toxicity and binding of the trivalent arsenic atoms to endogenous thiols, micromolar concentrations of the antidote 1,2-ethanedithiol (EDT_2) were included in the labeling solution. Double immunofluorescence staining on HEK293 cells expressing the α1(TetraCys)β2γ2 combination was performed using the biarsenical fluorophores and an antibody selectively recognizing the α1 subunit. The α1 subunit-selective antibody served as a control for the specificity of the Lumio staining. Although we observed high background staining with Lumio green, in many HEK293 cells expressing α1(TetraCys)β2γ2 the labeling matched very well the α1 antibody staining at the cell surface and at intracellular sites (Fig. 3). However, also non-transfected HEK293 cells exhibited considerable staining with Lumio green (not shown). In contrast, the second fluorophore Lumio red showed no colocalization with α1 antibody labeled receptors and was nonspecifically distributed throughout the cells (Fig. 3). All efforts to optimize the labeling conditions and to reduce background labeling, including labeling time, EDT_2 concentration, Lumio dye concentrations and washing conditions, did not result in any improvement of specific labeling. Therefore, this, at first sight, very promising labeling method could not be used for the planned studies on the trafficking of α5-$GABA_A$ receptors, which required pulse-chase experiments.

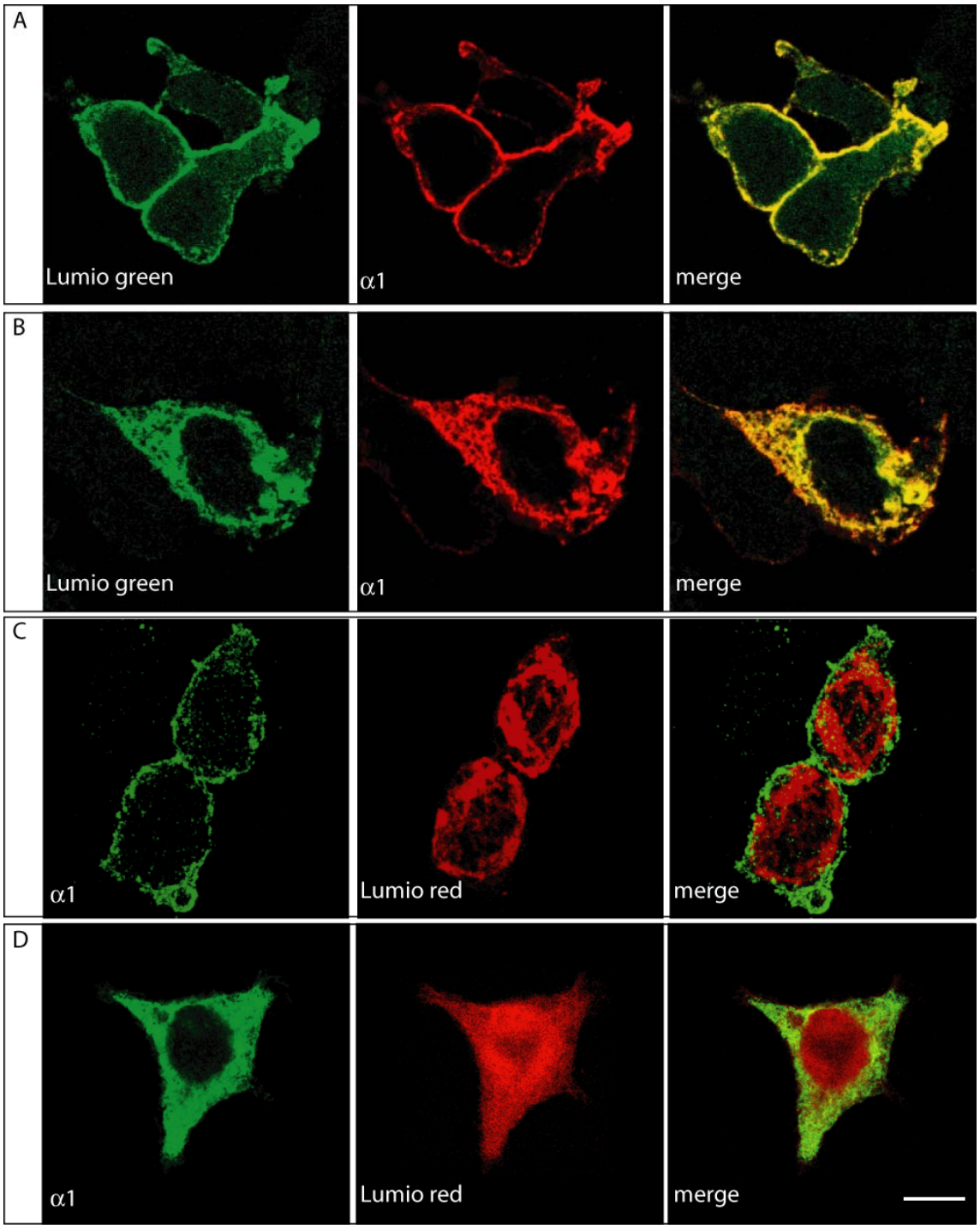

Figure 3. Specificity of Lumio green and Lumio red biarsenical dyes tested on HEK293 cells expressing α1(TetraCys)β2γ2 $GABA_A$ receptors. Live cells expressing the α1(TetraCys)β2γ2 combination were labeled with Lumio green (1 μM) or Lumio red (2.5 μM), which bind to the TetraCys tag within the α1 subunit. Immunoflourescence staining with α1 specific antibodies was used to test for the specificity of Lumio dye labeling. (A) Nonpermeabilized cells as well as permeabilized cells (B) showed a good colocalization of Lumio green (green) and α1 antibody staining (red). On the contrary, no colocalization of α1 staining (green) has been achieved with Lumio red in either nonpermeabilized (C) or in permeabilized cells (D). In all cases a high background level was encountered. Scale bar 10 μm.

Since different cell types may yield distinct levels of background labeling and because the efficient labeling of tagged AMPA receptors transiently expressed in neurons with biarsenical dyes has been reported (Ju *et al*. 2004), we next tried to apply this method to transfected primary cultured cortical neurons. In order to discriminate transfected α1TetraCys subunits from the endogenous α1 subunits present in neurons, we added a myc-tag or a flag-tag at the C-terminus of the α1TetraCys subunit. Transfection of the engineered α1 subunit into cultured neurons was achieved with nucleofection. Since this transfection method requires a sustained expression of the vector protein over a period of one to three weeks, the α1TetraCys-myc or α1TetraCys-flag constructs were subcloned into the appropriate expression vector pβAct. The pβAct vector contains the chicken β-actin promoter and enables moderate and sustained protein expression, but yielded rather low transfection efficiencies (less than 1%). The specificity of Lumio dyes was tested by immunoflourescence staining of nontransfected neurons and neurons transfected with the α1TetraCys-myc or the α1TetraCys-flag construct. Live nontransfected neurons were incubated with Lumio dyes, fixed and stained with α1 antibody that binds to endogenously expressed α1 subunits. Unfortunately, both Lumio dyes caused high background levels as well as significant cytotoxicity, as indicated by a vacuolized cell soma (Fig. 4).

Furthermore, transfected neurons were also labeled with Lumio dyes, fixed and probed for the presence of transfected and endogenous α1 subunits using antibodies that recognize the myc-tag, flag-tag or α1 subunit. As in nontransfected neurons, both Lumio green and Lumio red dyes exhibited considerable nonspecific staining in neurons (Fig. 5). Neurons had severely vacuolized soma and the Lumio dyes did not specifically colocalize with myc, flag or α1 antibody signals. All attempts to reduce the background staining to a manageable level failed. Therefore this labeling method could not be used for the intended experiments.

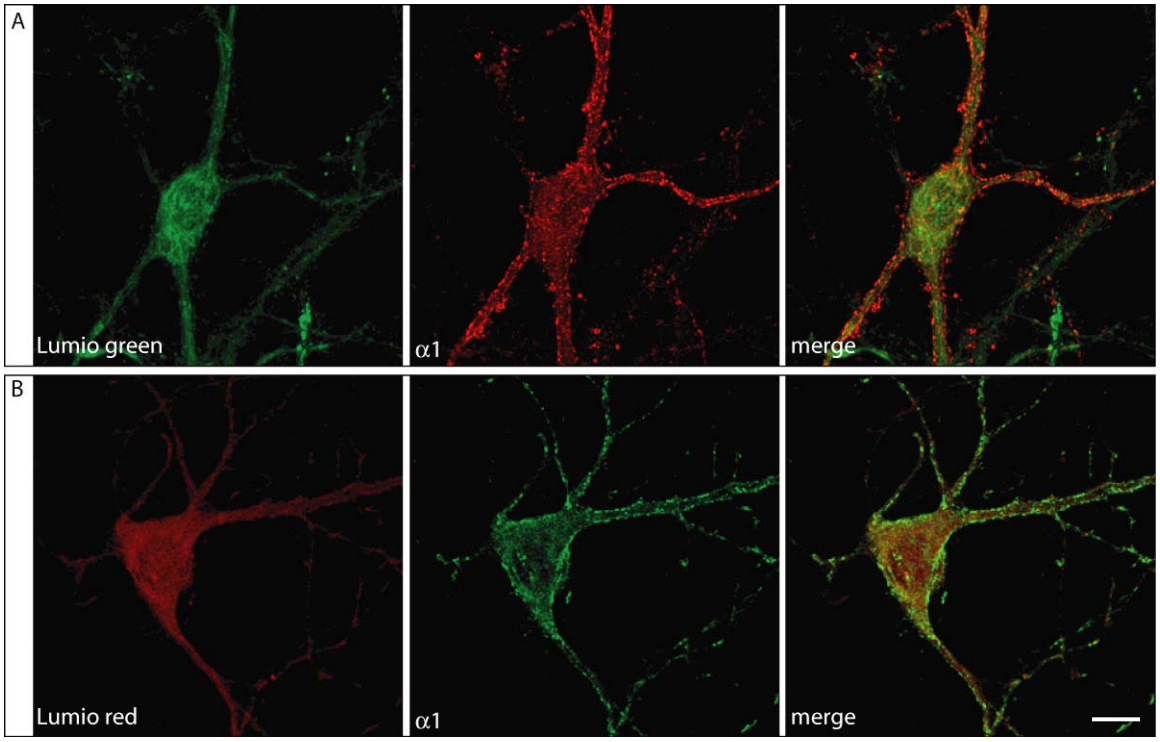

Figure 4. Lumio green and Lumio red biarsenical dyes tested on nontransfected cultured cortical neurons demonstrate severe background levels. Living neuronal cells (DIV21) were labeled with Lumio green (1 μM) or Lumio red (2.5 μM) and, upon fixation with PFA and permeabilization with Triton Tx-100, immunostained with α1 specific antibodies. (A) Lumio green and (B) Lumio red labeled nonspecifically neurons. Control staining for the α1 subunit, mostly found in the plasma membrane, (A in red, B in green) showed that Lumio dyes accumulated in the soma. Scale bar: 10 μm.

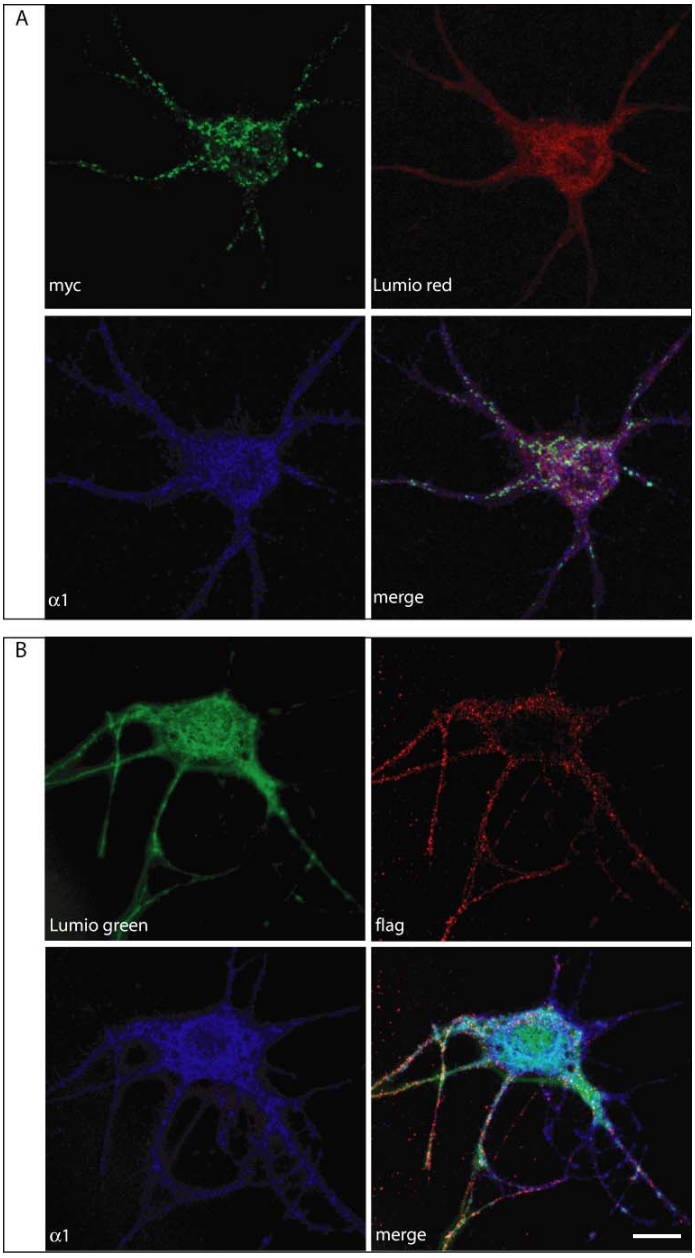

Figure 5. Specificity of Lumio dyes tested on cortical neurons expressing α1TetraCys-myc or α1TetraCys-flag constructs. Neurons were transfected using the nucleofection method with the α1TetraCys subunit tagged with the myc or flag epitope to enable the distinction between transfected and endogenous α1 subunits. Live cells (DIV8) were labeled with Lumio red (2.5 μM) or Lumio green (1 μM), fixed with PFA, permeabilized with Triton X-100 and immunostained using α1 and myc or flag antibodies. (A) Labeling of the α1TetraCys-myc tagged subunit with myc antibodies (green), Lumio red (red) and α1 antibodies (blue). (B) Labeling of the α1TetraCys-flag subunit with flag antibodies (red), Lumio green (green) and α1 antibodies (blue). Myc and flag antibodies selectively stained the TetraCys tagged α1 subunit, whereas the α1 antibody detected both the transfected and endogenous α1 subunit. In both cases, Lumio staining did not colocalize with myc or flag antibody staining and therefore exhibited exclusively non-specific staining. Scale bar: 10 μm.

6.5 Imaging of $GABA_A$ receptors in HEK293 cells and cultured cortical neurons using α-bungarotoxin binding site-tagged α5 subunits

To circumvent the problem of high background staining with Lumio dyes we aimed for another promising approach to monitor $GABA_A$ receptor trafficking within living cells. This new labeling method is based on the introduction of the minimal α-bungarotoxin binding site into the N-terminal domain of the α5 subunit. α-Bungarotoxin is a high-affinity ligand for the nicotinic acetylcholine receptor and binds to the short amino acid sequence WRYYESSLEPYPD in a virtually irreversible manner (Harel *et al*. 2001). It is available in a fluorescent, biotinylated and radioactive form, which enables a large variety of quantitative and qualitative analyses of the tagged protein, including pulse-chase experiments. In addition, this labeling method has been successfully applied on transfected neurons (Sekine-Aizawa and Huganir 2004).

The α-bungarotoxin tag (btx) was introduced into the extracellular domain of the α5 subunit (amino acid 36) to permit the selective labeling of cell-surface receptors and analysis of receptor membrane insertion as well as receptor internalization. The labeling method was first established on HEK293 cells expressing α5btxβ3γ2 receptors. Living cells were treated with AlexaFlour488-conjugated α-bungarotoxin to label the α5btx subunit. After fixation, the specificity of α-bungarotoxin labeling was tested using immunostaining with α5 and β2/3 specific antibodies. In HEK293 cells excellent colocalization of AlexaFlour488-α-bungarotoxin and α5 antibody signals was found (Fig. 6). On nontransfected HEK293 cells, AlexaFlour488-α-bungarotoxin showed virtually no background staining. Since the specificity of AlexaFlour488-α-bungarotoxin labeling of α5btx subunit was confirmed, we focused on establishing this method in neurons to study the trafficking of α5-$GABA_A$ receptors.

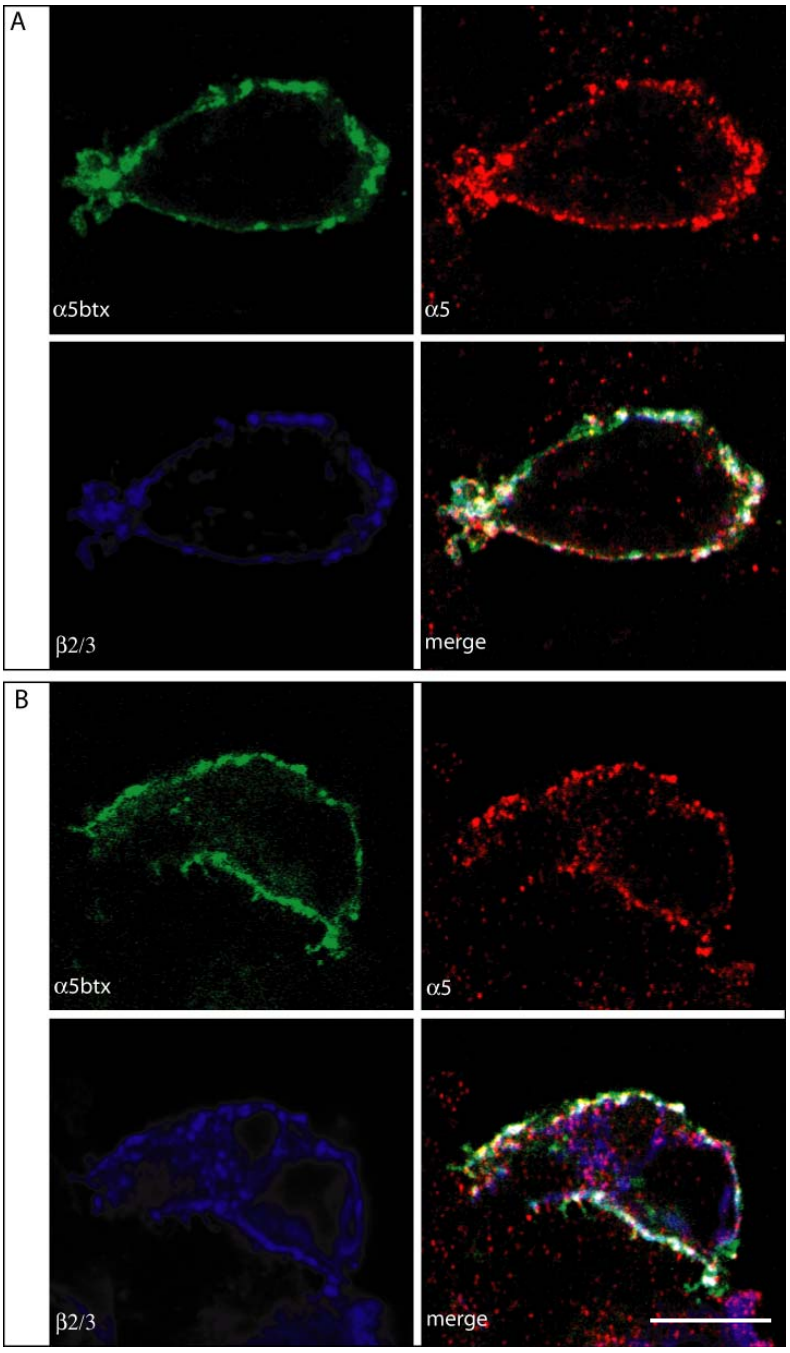

Figure 6. AlexaFlour488-α-bungarotoxin labeling of α5btxβ3γ2 receptors expressed in HEK293 cells. AlexaFlour488-α-bungarotoxin labeling of the α5btx subunit was performed on living cells, which were subsequently fixed and double immunostained using α5 and β2/3 specific antibodies. (A) Nonpermeabilized cells showed colocalization (white) of α5 (red) and β3 (blue) immunostaining with AlexaFlour488-α-bungarotoxin labeling of the α5btx subunit (green) at the cell surface. (B) Permeabilized cells revealed in addition low levels of intracellular staining. Scale bar:10 μm.

After successful application of the α-bungarotoxin labeling method on α5btxβ3γ2 receptor expressing HEK293 cells, we applied this technique on cultured neurons transfected with the α5btx subunit construct. Cortical neurons (DIV 4) were transfected using the NeuroFect agent, which - in our hands - exhibited higher transfection efficiency than the nucleofection method. After 24 hours of expression, neurons were briefly incubated with tubocurarine to block endogenous α-bungarotoxin binding sites present on α7 nicotinic acetylcholine receptors. Subsequently, nontransfected neurons and neurons transfected with the α5btx construct were both labeled with AlexaFlour488-α-bungarotoxin to determine nonspecific and specific labeling signals, respectively. As a control, neurons were immunostained for the presence of α5 subunits. Unfortunately, the AlexaFlour488-α-bungarotoxin labeling did not colocalize with the α5 antibody staining in transfected neurons (Fig. 7). Moreover, the transfected and nontransfected cortical neurons were essentially indistinguishable and showed high background levels upon labeling with AlexaFlour488-α-bungarotoxin.

In contrast to HEK293 cells, AlexaFlour488-α-bungarotoxin displayed considerable non-specific staining in neurons, even after blocking endogenous α-bungarotoxin binding sites with tubocurarine. Therefore, this labeling method was not suitable for our intended studies on trafficking of α5(H105R) receptors in neurons.

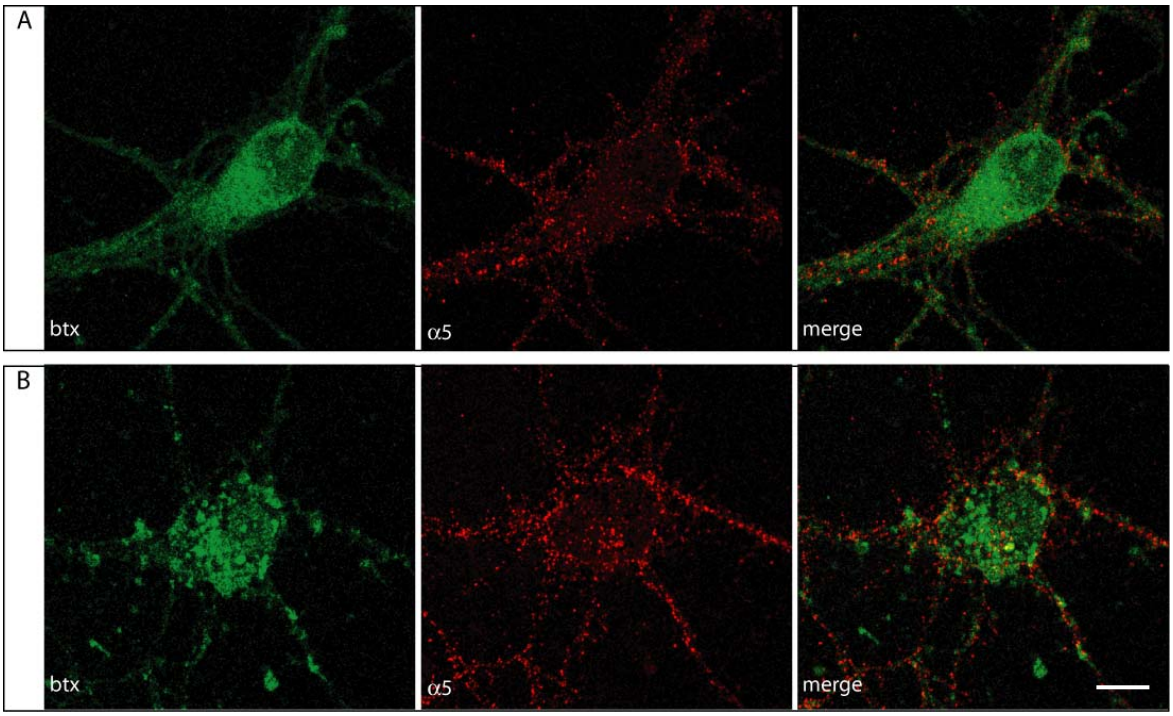

Figure 7. Specificity of α-bungarotoxin labeling tested on nontransfected and on α5btx transfected cultured cortical neurons. After preincubation with tubocurarin, live cortical neurons (DIV 5) were labeled with AlexaFlour488-α-bungarotoxin (green), fixed and immunostained with α5 antibody (red). (A) Nontransfected neurons showed significant background when stained with AlexaFlour488-α-bungarotoxin. (B) Neurons transfected with the α5btx construct using the NeuroFect reagent, were also nonspecifically labeled with AlexaFlour488-α-bungarotoxin. Scale bar: 10 μm.

6.6 Expression and targeting of α5 and α5(H105R)-GFP-tagged subunits in primary cortical neurons

The approaches of labeling tetracysteine or α-bungarotoxin tagged α5 subunits with Lumio dyes and AlexaFlour488-α-bungarotoxin conjugate, respectively, were not suitable for monitoring trafficking of the receptors in neurons due to high nonspecific labeling. Therefore, we used the well established GFP tag to label the α5 subunit, which was expected to answer the question to what extent α5(H105R) subunits are expressed in neurons and targeted into dendrites.

The GFP tag was inserted at the C-terminus of the wild type α5 subunit and subsequently the H105R mutation was introduced by site directed mutagenesis. Using NeuroFect, cultured cortical neurons were transfected with plasmids containing either the α5-GFP or the α5(H105R)-GFP construct. After 24 hours, the neurons were fixed and probed for α5- and GFP-immunoreactivity (Fig. 8). The GFP tag enabled immediate detection of the transfected α5 subunit protein, while GFP and α5 subunit antibodies were used to confirm the specificity of the signals. Analysis by confocal scanning microscopy showed that the GFP-tagged α5 and α5(H105R) subunits were expressed to similar levels in the soma of neurons and were both targeted into proximal and distal dendrites. The GFP fluorescence colocalized with GFP and α5 subunit antibody staining, documenting the specificity of the signals. Since no appreciable difference in expression and targeting was observed, these processes did not seem to be significantly influenced by the H105R mutation upon over-expression in neurons.

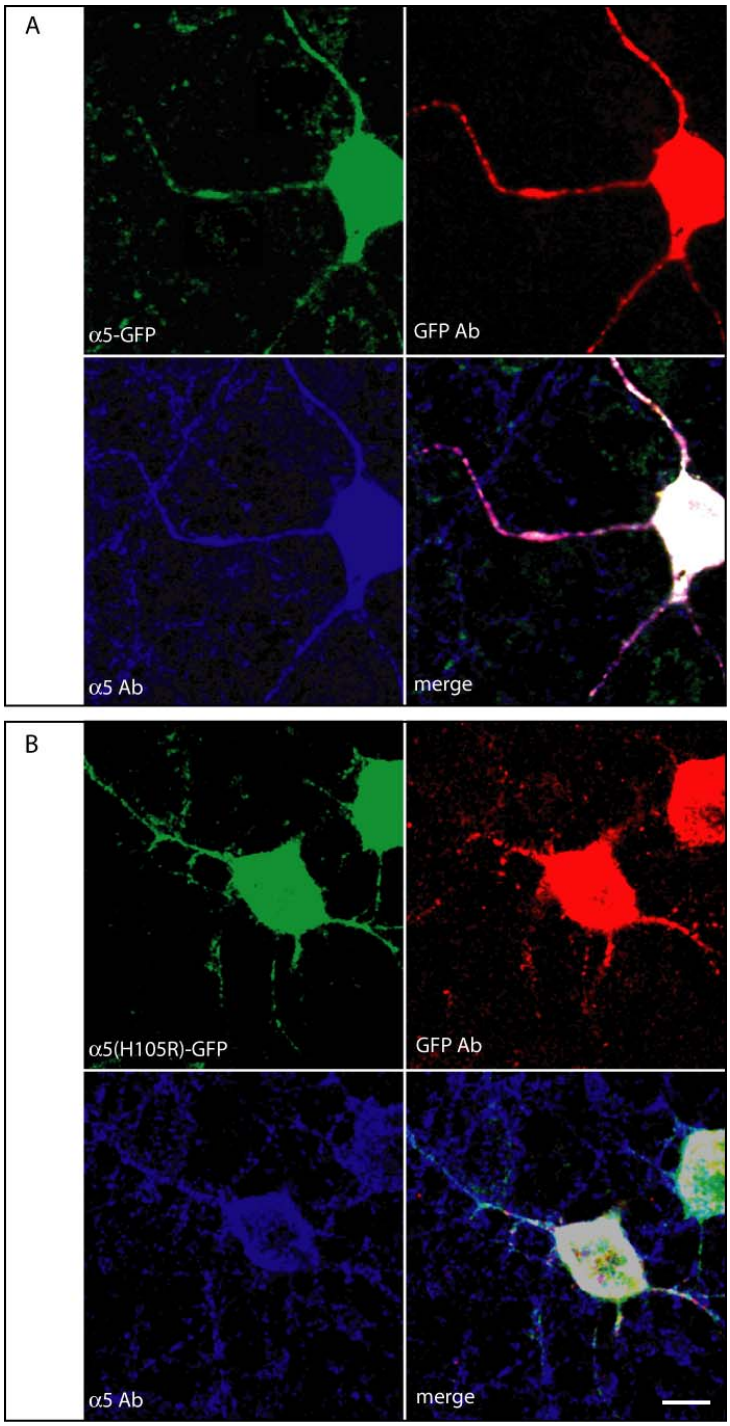

Figure 8. Expression and targeting of GFP-tagged α5 and α5(H105R) subunit in cultured cortical neurons. Cortical neurons (DIV5) were transfected with the α5-GFP or α5(H105R)-GFP construct using NeuroFect and analyzed 24 h later. Cells were fixed, permeabilized and double immunostained using α5 (blue) and GFP (red) specific antibodies, which showed colocalization (white) with the GFP signal (green). The α5 antibody staining has detected endogenous α5 subunits as well. No apparent differences in expression and targeting between (A) α5-GFP and (B) α5(H105R)-GFP subunits were observed. Scale bar: 10 μm.

6.7 Determination of benzodiazepine binding sites in the hippocampus of α5(H105R) mice using receptor autoradiography

The results accumulated so far indicated that although the H105 mutation caused a moderate decline in α5 subunit protein levels *in situ*, the expression and targeting of α5(H105R) subunits appeared to be unchanged after overexpression in HEK293 cells as well as in cultured neurons. However, the limitation of antibody-based methods to analyze heterooligomeric proteins is that total subunit protein levels are monitored, irrespective of whether they are assembled into functional protein complexes or not. Since binding of ligands to the benzodiazepine site requires fully assembled $GABA_A$ receptors, radioligand binding provides a means to analyze for the presence of assembled receptor complexes. To determine the levels of assembled α5-$GABA_A$ receptors in brain tissue of wild type and α5(H105R) mice, two radioligands interacting with the benzodiazepine site were used: [^{3}H]Ro15-4513, which binds to all $GABA_A$ receptor subtypes containing an α subnit and the γ2 subunit (Benke *et al*. 1996) and [^{3}H]L655,708, which interacts with high affinity selectively with the α5-$GABA_A$ receptor subtype (Quirk *et al*. 1996, Casula *et al*. 2001).

To achieve a high spatial resolution, parasagittal brain sections were probed for [^{3}H]Ro15-4513 binding using a saturating concentration of radioligand. When inspected visually, autoradiograms of brain sections of wild type and α5(H105R) knock-in mice appeared to display similar [^{3}H]Ro15-4513 binding levels (Fig. 9). However, quantification of [^{3}H]Ro15-4513 binding to the whole hippocampal formation revealed a 7 ± 0.5 % ($n = 9$, $P < 0.02$, two-tailed t-test) reduction of [^{3}H]Ro15-4513 binding in α5(H105R) mice, which is attributed to the deficit of α5-$GABA_A$ receptors in the mutants. Since in the hippocampus of wild type mice α5-$GABA_A$ receptors account for 22 ± 2 % of $GABA_A$ receptors (Sur *et al*. 1998), this finding implies an about 30-40% reduction of α5(H105R)-$GABA_A$ receptors. A detailed quantification of [^{3}H]Ro15-4513 binding levels in individual regions of the hippocampal formation revealed a non-uniform 8-18 % reduction of binding, depending on the subregion (Tab. 2). The most prominent reduction in [^{3}H]Ro15-4513 binding was detected in CA1 stratum oriens, CA1 stratum lacunosum, CA3 stratum radiale and dentate gyrus granule cell layer (Tab. 2).

In order to directly quantify α5-$GABA_A$ receptor levels on parasagittal brain sections, we used the α5 subtype-specific radioligand [^{3}H]L655,708, which displays a 100-fold higher affinity for α5-$GABA_A$ receptors than for other $GABA_A$ receptor subtypes. Most strikingly, a strong reduction of [^{3}H]L655,708 binding was observed in all brain areas expressing the α5 subunit (Fig. 9). Quantification of [^{3}H]L655,708 binding levels revealed a reduction of 39 ± 4 % in the olfactory bulb ($P < 0.0001$), 47 ± 5% in the hippocampal formation ($P < 0.0001$), 43 ± 0.4 % in the superior colliculus ($P < 0.0001$) and 53 ± 6 % in the motor cortex ($P < 0.0001$, two-tailed t-test, n= 6 mice**,** 3 sections per mouse). These findings indicate that the strong reduction of α5-$GABA_A$ receptors in α5(H105R) mice is not limited to hippocampus, but it is common to all brain regions expressing the α5-$GABA_A$ receptor subtype.

As for [^{3}H]Ro15-4513 binding levels, a detailed quantification revealed a non-uniform reduction (18-66 %) of [^{3}H]L655,708 binding among hippocampal subregions, with the highest reduction in CA1 stratum oriens, CA1 stratum pyramidale, and dentate gyrus granule cell layer (Tab. 2).

The reduction of [^{3}H]Ro15-4513 binding in the hippocampal subregions analyzed largely corresponded to that observed for [^{3}H]L655,708 binding. However, differences in the degree of reduction were found in the CA1 stratum pyramidale and in the hilus of the dentate gyrus, where a reduction of [^{3}H]Ro15-4513 binding was not clearly detected or moderate, respectively, whereas the [^{3}H]L655,708 binding was strongly decreased. At present the cause for these mismatches is unknown. Other minor inconsistencies are apparently due to variations in labeling intensities among sections, which is reflected by the partly large standard deviation observed for [^{3}H]Ro15-4513 binding.

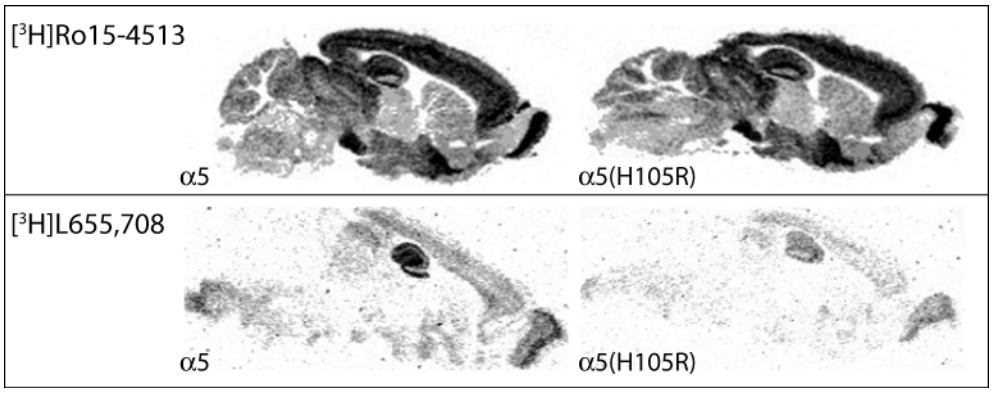

Figure 9. Autoradiographic distribution of [^{3}H]Ro15-4513 and [^{3}H]L-655,708 binding sites in parasagittal brain sections of wild type and α5(H105R) mice. Top panel: Labeling of diazepam-sensitive and diazepam-insensitive $GABA_A$ receptors with [^{3}H]Ro15-4513 (20 nM) in 12 μm parasagittal brain slices of wild type and α5(H105R) mice. Quantification of [^{3}H]Ro15-4513 binding in hippocampus revealed a 7 ± 0.5 % (n = 9, P< 0.02, two-tailed t-test) reduction of binding in α5(H105R) mice. Bottom panel: Labeling of α5-$GABA_A$ receptors using the α5-specific ligand [^{3}H]L655,708 (1 nM). Binding levels were strongly reduced in all brain areas expressing the α5 subunit of α5(H105R) mice. Quantification of [^{3}H]L655,708 binding in hippocampus revealed a 47 ± 5 % (n=6) reduction of binding in α5(H105R) mice. Nonspecific binding was assessed in the presence of 10 μM flumazenil and resulted in virtually no background staining at the exposure times used (not shown).

Table 2. Quantification of [^{3}H]Ro15-4513 and [^{3}H]L-655,708 binding in the hippocampal formation of wild type and α5(H105R) mice.

	[^{3}H]Ro15-4513 bound (nCi/mg wet tissue)				[^{3}H]L655,702 bound (nCi/mg wet tissue)			
Hippocampus region	α5	α5(H105R)	% change	P	α5	α5(H105R)	% change	P
CA1 s. or	16.31 ± 0.34	13.55 ± 0.20	- 17 ± 1	< 0.001	3.42 ± 0.31	1.44 ± 0.21	- 58 ± 2	< 0.0001
CA1 s. pyr	12.94 ± 0.31	12.72 ± 0.58	- 2 ± 2	N.S.	2.24 ± 0.24	0.78 ± 0.17	- 65 ± 4	< 0.0001
CA1 s. rad	17.25 ± 1.06	15.47 ± 0.28	- 10 ± 4	< 0.002	3.49 ± 0.32	1.66 ± 0.16	- 52 ± 0.2	< 0.0001
CA1 s. lm	16.46 ± 0.34	14.41 ± 0.06	- 12 ± 1	< 0.0001	3.04 ± 0.20	1.42 ± 0.13	- 53 ± 1	< 0.0001
CA3 s. or	12.53 ± 0.30	10.58 ± 1.35	- 16 ± 9	< 0.01	2.45 ± 0.39	2.00 ± 0.28	- 18 ± 2	< 0.0001
CA3 s. pyr	8.75 ± 0.06	8.05 ± 1.05	- 8 ± 11	< 0.01	1.76 ± 0.21	0.89 ± 0.17	- 50 ± 4	< 0.0001
CA3 s. rad	15.08 ± 1.60	12.39 ± 0.63	- 18 ± 5	< 0.0001	3.45 ± 0.26	1.86 ± 0.20	- 46 ± 2	< 0.0001
DG s. mol	19.66 ± 0.47	17.93 ± 0.14	- 9 ± 1	< 0.001	4.27 ± 0.43	2.31 ± 0.18	- 46 ± 1	< 0.0001
DG granule cell layer	10.22 ± 1.38	8.97 ± 1.32	- 12 ± 1	N.S.	1.87 ± 0.22	0.63 ± 0.10	- 66 ± 1	< 0.0001
DG, hilus	19.07 ± 0.93	18.06 ± 1.04	- 5 ± 1	N.S.	4.08 ± 0.32	2.27 ± 0.15	- 44 ± 1	< 0.0001

Radioligand binding was performed on fresh-frozen brain sections derived from 3 mice per genotype (3 sections per animal) for [^{3}H]Ro15-4513 binding and 6 mice per genotype (3 sections per animal) for [^{3}H]L655,708 binding. Images were acquired using a high resolution phosphoimaging system and signals were quantified using the OptiQuant software. Nonspecific binding was assessed using excess flumazenil. Values are expressed as mean ± SD (n=6). Data evaluated using two-tailed t-test. Abbreviations: s. or, stratum oriens; s. pyr, stratum pyramidale; s. rad, stratum radiale; s. lm, stratum lacunosum-moleculare; DG, dentate gyrus; s. mol, stratum moleculare; NS, non significant (P > 0.05).

6.8 Affinity and abundance [^{3}H]L655,708 binding sites in wild type and α5(H105R) knock-in mice

The receptor autoradiography revealed a strong reduction of high affinity [^{3}H]L655,708 binding to α5-GABA$_A$ receptors in the brain of α5(H105R) knock-in mice. Unlike [^{3}H]Ro15-4513, which was used at saturating concentrations (5-6 fold of K_D-value), [^{3}H]L655,708 could only be used at near-saturating concentrations (2 fold of K_D-value) since at higher ligand concentrations there is a risk of labeling also other GABA$_A$ receptor subtypes. Therefore, moderate changes in the affinity to [^{3}H]L655,708 potentially caused by the H105R mutation in the α5(H105R) receptors could result in the observed reduction in [^{3}H]L655,708 binding. To rule out any effect of the H105R mutation on the affinity of [^{3}H]L655,708, saturation binding experiments were performed to determine the affinity (K_D value) and number of binding sites (B_{max} value) of [^{3}H]L655,708 for wild type and mutant α5-receptors. Saturation binding experiments were done on crude membranes prepared from brains of wild type and α5(H105R) mice using increasing concentrations of [^{3}H]L655,708. As expected, the binding data fitted best to a model of two binding sites for [^{3}H]L655,708 (Fig. 10): a high affinity binding site corresponding to α5-receptors and a low affinity binding site corresponding to other receptor subtypes (Quirk *et al*. 1996). Analysis of the high affinity component revealed a 76 ± 10% reduction (n=4, $P < 0.0001$, two tailed t-test) of binding sites for [^{3}H]L655,708 in α5(H105R) mice (B_{max1} = 9 ± 3 fmol/mg protein) as compared to wild type mice (B_{max1} = 39 ± 6 fmol/mg protein) with no change of the affinity (K_{D1} α5(H105R): 0.6 ± 0.2 nM, K_{D1} wild type 0.5 ± 0.1 nM). In contrast, K_D and B_{max} values of the low affinity binding component were similar in both genotypes (α5(H105R): K_{D2} = 44 ± 6 nM and B_{max2} = 505 ± 190 fmol/mg, wild type: K_{D2} = 58 ± 3 nM and B_{max2} = 448 ± 193 fmol/mg). These results demonstrate that the number of binding sites and not the affinity of α5(H105R)-GABA$_A$ receptor for [^{3}H]L655,708 is affected by the α5(H105R) mutation.

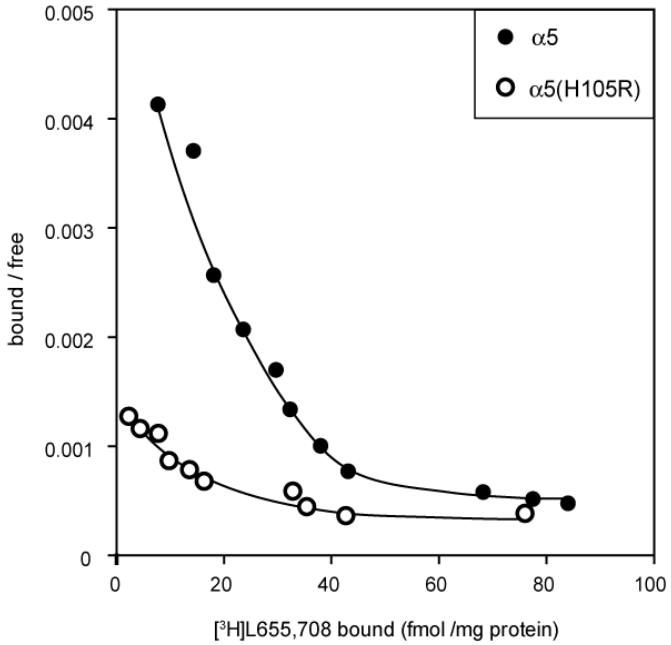

Figure 10. Scatchard representation of [^{3}H]L655,708 saturation binding data to wild type and α5(H105R) brain membrane preparations. The binding of [^{3}H]L655,708 displays two components: the high affinity site represents binding to α5-GABA$_A$ receptors and low affinity site represents binding to other GABA$_A$ receptor subtypes. The high affinity component is characterized in wild type mice by K_{D1} = 0.5 ± 0.1 nM and B_{max1} = 39 ± 6 fmol/mg protein. In α5(H105R) mice the K_D was unchanged (K_{D1} = 0.6 ± 0.2 nM) but the number of binding sites was strongly reduced (B_{max1} = 9 ± 3 fmol/mg protein). The low affinity components were similar in both genotypes (wild type: K_{D2} = 58 ± 3 nM, B_{max2} = 448 ± 193 fmol/mg protein; α5(H105R): K_{D2} = 44 ± 6 nM, B_{max2} = 505 ± 190 fmol/mg protein). The results shown are representative for three independent experiments.

6.9 Analysis of the amount of assembled α5(H105R)-GABA$_A$ receptors

The saturation binding experiments revealed a strong reduction (76%) of high affinity binding sites for [^{3}H]L655,708, corresponding to α5-GABA$_A$ receptors in α5(H105R) mice. However, this finding did not correspond to the moderate 23 ± 2% reduction of the α5(H105R) subunit protein. Thus, we hypothesized that α5 subunits containing the H105R mutation may not efficiently assemble into functional, i. e. ligand binding receptor complexes. In order to separate potential unassembled α5 subunits from assembled α5-receptor complexes, we performed sucrose density gradient analyses on brain extracts of α1(H101R) and α5(H105R) mice. Brain extracts of α1(H101R) mice

served as a control, since the analogues mutation in the α1 subunit did not influence the expression levels of this $GABA_A$ receptor subtype (Rudolph *et al*. 1999). Brain membrane proteins of both genotypes were solubilized and subsequently separated according to their molecular sizes on continuous 5-20 % sucrose density gradients. After centrifugation, the gradients were fractioned and individual fractions were analyzed for the presence of α5, α1, β2/3 and γ2 subunits by Western blotting (Fig. 11 A, B). In α1(H101R) mice the α1-, α5-, β2/3- and γ2-signals were detected in the high molecular fractions, indicating that the entire population of $GABA_A$ receptor subunits assembled into macromolecular receptor complexes. Likewise, in α5(H105R) mice, all subunits were detected as well in the high molecular fractions, also indicating the presence of assembled α5(H105R)-$GABA_A$ receptors. No evidence for appreciable amounts of monomeric, non-assembled α5 subunits was found. The signal intensity of α5 subunits on the Western blots derived from gradients of α5(H105R) mice was found to be lower than the staining intensity of α5 subunits in the blots derived from gradients of α1(H101R) mice (Fig. 11 A, B), being in line with the reduced expression levels of α5(H105R) subunits.

The gradient fractions were also probed for their ability to bind [^{3}H]Ro15-4513 and [^{3}H]L655,708. As expected, a peak of binding for both radioligands was detected in the high molecular weight fractions of both genotypes. However, whereas both genotypes displayed almost identical binding profiles for [^{3}H]Ro15-4513 (Fig. 9 C), [^{3}H]L655,708 binding was strongly reduced (60 ± 6.6 %, n=3) in gradients derived from α5(H105R) mice (Fig. 11 D). These results indicate that α5 subunits containing the H105R mutation assemble into high molecular complexes and display strongly reduced [^{3}H]L655,708 binding.

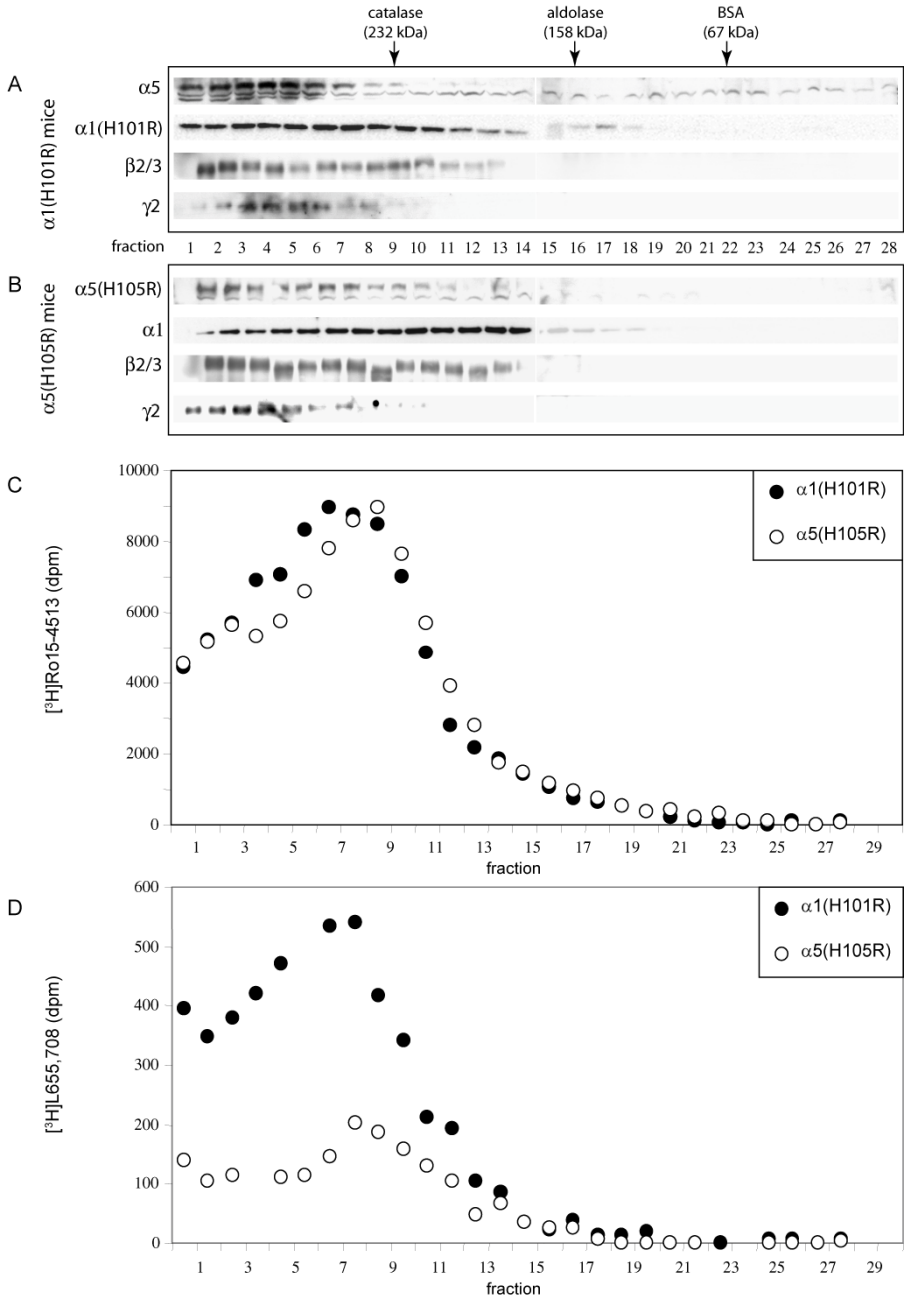

Figure 11. Sucrose density gradient centrifugation of native $GABA_A$ receptors from brains of α1(H101R) and α5(H105R) mice. Deoxycholate extracts of mouse brain membranes were separated according to their molecular size on 5-20 % linear sucrose gradient by centrifugation at 170 000 x g for 15 h at 4°C. (A, B) After centrifugation, individual fractions were analyzed for α1, α5, β2/3 or γ2 subunit immunoreactivity by Western blotting. (A) Western blots of gradient fractions derived from brain membrane extracts of α1(H101R) mice, which were used as a control. (B) Western blots of gradient fractions derived from brain membrane extracts of α5(H101R) mice. In both genotypes, all subunits were exclusively detected in the high molecular fractions, indicating assembled $GABA_A$ receptor complexes. The α5(H105R) subunit was found to be less abundant than the α5 subunit in α1(H101R) mice as judged by their signal intensities. Molecular size calibration was achieved using marker proteins (bovine serum albumine, aldolase and catalase) run in parallel gradients. (C, D) Determination of [^{3}H]Ro15-4513 (12 nM) and [^{3}H]L655,708 (2 nM) binding present in the fractions of the gradients containing α1(H101R) and α5(H105R) brain extracts. The data shown are representative for three independent experiments.

6.10 Pharmacological separation of $GABA_A$ receptor subtypes in wild type and α5(H105R) knock-in mice

So far, the severe loss in α5(H105R) mice of high affinity [^{3}H]L655,708 binding sites cannot be explained by a fundamentally impaired assembly or targeting of α5 receptors. We neither observed the accumulation of an intracellular pool of unassembled α5(H105R) subunits in brain tissue nor an impaired expression and cell surface targeting after over-expression of α5(H105R) subunits in either HEK293 cells or primary cultured neurons. However, more subtle alterations may occur that resulted in the observed loss of α5-selective binding. In this respect, the existence of $GABA_A$ receptors containing two different types of α subunits in a single receptor complex has been well documented (Araujo *et al*. 1999, Del Rio *et al*. 2001, Benke *et al*. 2004). In such receptor complexes, the benzodiazepine site binding properties is expected to depend on the positioning of the distinct α subunits within the complex since the interface of α and γ2 subunits forms the binding site (reviewed by Siegel 2002). Thus, only the type of α subunit positioned next to the γ2 subunit is expected to contribute to the benzodiazepine site and would determine its drug binding properties. In line with this hypothesis, in receptors containing the α1 and α5 subunit, the α5 subunit was found to be pharmacologically dominant over the α1 subunit and consequently the receptor displayed α5 pharmacology (Araujo *et al*. 1999). The predominance of α5-pharmacology implicates that during assembly of the receptor complex the α5 subunit is favorably arranged next to the γ2 subunit. Therefore, we hypothesized that the introduction of the H105R mutation into the α5 subunit might influence the arrangement of the α5 subunit within α1α5(H105R)-$GABA_A$ receptors. If in such receptor complexes the α1 subunit would preferentially be located next to the γ2 subunit, this would provide an explanation for the loss of α5-specific [^{3}H]L655,708 binding sites.

We aimed to test this hypothesis using radioligand competition experiments with the ligands [^{3}H]Ro15-4513 and zolpidem as displacing agent. Zolpidem discriminates by affinity major $GABA_A$ receptor subtypes. It displays high affinity (10-20 nM) for receptors containing the α1 subunit, intermediate affinity for α2- and α3- containing receptors (200-300 nM) and low affinity for α5-containing receptors (6->10 μM) (Ruano *et al*. 1992, Benavides *et al*. 1993, Quirk *et al.* 1996). The presence and

abundance of zolpidem binding sites in brain tissue of wild type and α5(H105R) mice was determined by competing the binding of [^{3}H]Ro15-4513 ligand with increasing concentrations of zolpidem (0.5 nM to 100 μM). As expected, the binding data fitted best to a model consisting of three distinct binding sites for zolpidem in both genotypes (Fig. 12). No apparent change in the affinity and abundance of the high (α1-receptors) and medium (α2- and α3-receptors) affinity sites for zolpidem was detected in α5(H105R) knock-in mice (Tab. 3, 4). However, the affinity of the low affinity binding site corresponding to α5-receptors was slightly decreased (wild type: 33 ± 9 μM, α5(H105R): 101 ± 32 μM, Tab. 3). This decrease in affinity was expected since the α5(H105R) mutation renders the α5 receptors largely insensitive to benzodiazepine full agonists (Benson *et al*. 1998). Furthermore, the number of low affinity, i. e. α5-receptor, binding sites was considerably lower (37%) in α5(H105R) mice (B_{max3} = 264 ± 42 fmol/mg protein) than in wild type mice (B_{max3} = 403 ± 69 fmol/mg protein, Tab. 3)**.** Likewise, when the relative B_{max3} values within each genotype were calculated, we detected 12 % low affinity zolpidem sites in wild type and 8 % in α5(H105R) mice (Tab. 4, $P < 0.001$, two-tailed t-test). In this case, the relative abundance of receptors with α5 pharmacology in α5(H105R) mice would be reduced by 44 % as compared to the wild type mice.

A decrease in the number of low affinity zolpidem sites might be expected to be accompanied by an increase in high and/or medium affinity site if the H105R mutation indeed abolishes the potential predominant position of the α5 subunit next to the γ2 subunit. In all three experiments performed we observed a small increase in the relative abundance of high affinity zolpidem binding sites corresponding to the α1-subunit pharmacology (Tab. 4). Unfortunately, this small increase in high affinity zolpidem sites was masked by the variation among experiments. This was not surprising given the small population of α5-$GABA_A$ receptors containing two distinct types of α subunits. Nevertheless, the reduction of low affinity zolpidem binding sites and the concomitant increase in high affinity binding sites that was observed in each individual experiment suggests a change in the positioning of α subunits within $GABA_A$ receptors containing two distinct α subunits.

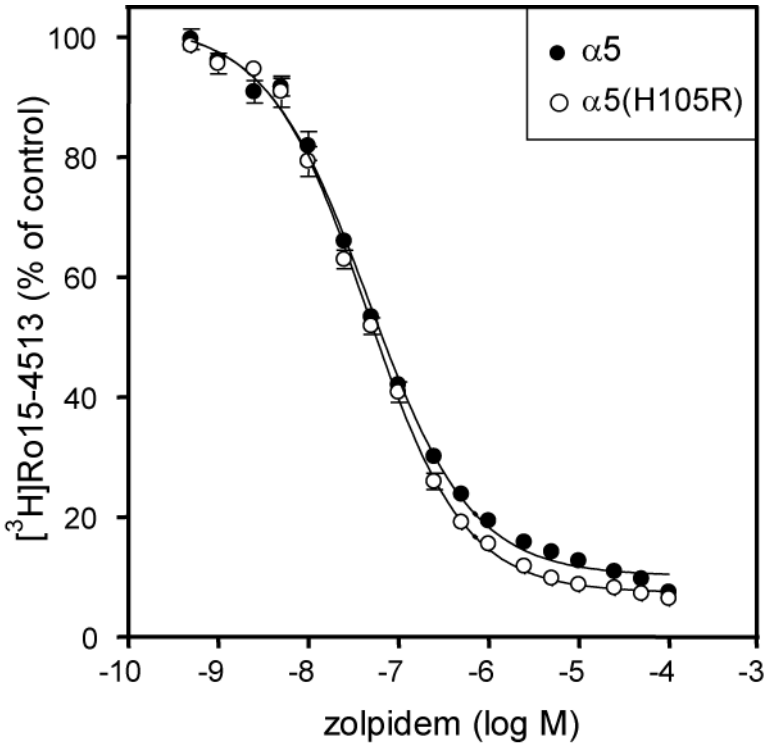

Figure 12. Zolpidem competition of [^{3}H]Ro15-4513 binding in wild type and α5(H105R) forebrain preparations revealed a reduced number of zolpidem-insensitive binding sites in α5(H105R) mice. Binding of [^{3}H]Ro15-4513 (6 nM) to forebrain preparations of wild type and α5(H105R) mice was displaced using increasing concentrations of zolpidem (0.5 nM to 100 μM). The number of high and medium affinity zolpidem binding sites was comparable in both genotypes. The relative number of zolpidem-insensitive binding sites in α5(H105R) mice was 44 % reduced as compared to wild type mice. Nonspecific binding was determined in presence of 10 μM flumazenil. Data represent the mean ± SD of three independent experiments. Standard deviations are not visible if smaller than the depicted circles. Ligand binding data were analyzed using the program 'KELL for Windows 6.0.5' (Biosoft, UK) and depicted in tables 3 and 4.

Table 3. Zolpidem binding to GABA$_A$ receptor subtypes in the forebrain of α5 wild type and α5(H105R) mice.

	K_{D1} (nM)	K_{D2} (nM)	K_{D3} (μM)	B_{max1} (fmol/mg)	B_{max2} (fmol/mg)	B_{max3} (fmol/mg)
α5	8.2 ± 0.9	124 ± 28	33 ± 9	2463 ± 229	433 ± 29	403 ± 69
α5(H105R)	9.0 ± 1.4	130 ± 48	101 ± 32	2563 ± 75	447 ± 99	264 ± 42

Values are means ± SD of three independent experiments and were derived form the competition experiments shown in Fig. 12.

Table 4. Relative abundance of zolpidem binding components in α5 wild type and α5(H105R).

	B_{max1} (%)	B_{max2} (%)	B_{max3} (%)
α5	76 ± 3	13 ± 2	12 ± 1
α5 (H105R)	80 ± 4	14 ± 3	8 ± 1

Values are means ± SD of three independent experiments and were derived form the competition experiments shown in Fig. 12. B_{max3} is significantly lower in the α5(H105R) mice ($P < 0.002$, two-tailed t-test).

6.11 Influence of the H105R mutation on the pharmacological dominance of the α5 subunit in α5-$GABA_A$ receptors containing two distinct types of α subunits

The zolpidem competition experiments were unfortunately not sensitive enough to provide clear evidence for the hypothesis that the H105R mutation may affect the positioning of the α5 subunit in $GABA_A$ receptors containing two distinct types of α subunits. To overcome this problem, we intended to immunoprecipitate the α5-$GABA_A$ receptor subtypes for a further analysis. However, one major shortcoming was the lack of sufficient amounts of α5 subunit-selective antibodies suitable for immunoprecipitation of α5-receptors.

Since we could not directly immunoprecipitate the α5 subunit containing receptors we made use of other $GABA_A$ receptor antisera to analyze the population of receptors containing the α5 subunit in addition to another type of α subunit. Among α5-receptors containing two different types of α subunits the α1α5-combination has been found to be most abundant (20% of α5 receptors, Araujo *et al*. 1999). Therefore, we examined the drug binding properties of α1-$GABA_A$ receptors immunopurified from wild type and α5(H105R) knock-in mice. To test for α5 subunit pharmacology in the receptors immunoprecipitated with the α1 antiserum, the immunoprecipitates were probed for α5-selective [^{3}H]L655,708 binding and for [^{3}H]Ro15-4513 binding as a control for the level of immunoprecipitated receptors. There was no significant difference ($P > 0.5$, Mann Whitney test) in the amount of [^{3}H]Ro15-4513 binding sites immunoprecipitated with the α1 antiserum from wild type (101 ± 10 fmol/mg protein) and α5(H105R) mice

(99 ± 7 fmol/mg protein, Fig. 13A). However, [^{3}H]L655,708 binding to the anti-α1 precipitated receptors was 49 ± 6 % reduced ($P < 0.02$, Mann Whitney test) in α5(H105R) knock-in mice (1.49 ± 0.35 fmol/mg protein) as compared to wild type mice (2.83 ± 0.36 fmol/mg protein, Fig. 13 B).Since [^{3}H]Ro15-4513 binding levels in the α1-immunoprecipitate derived from α5(H105R) mice were unchanged but [^{3}H]L655,708 binding levels were reduced by about 50% this result indicates that in α1α5(H105R) receptors the α5 subunit lost its pharmacological prevalence and may have an equal chance to occupy the position next to the γ2 subunit as the α1 subunit.

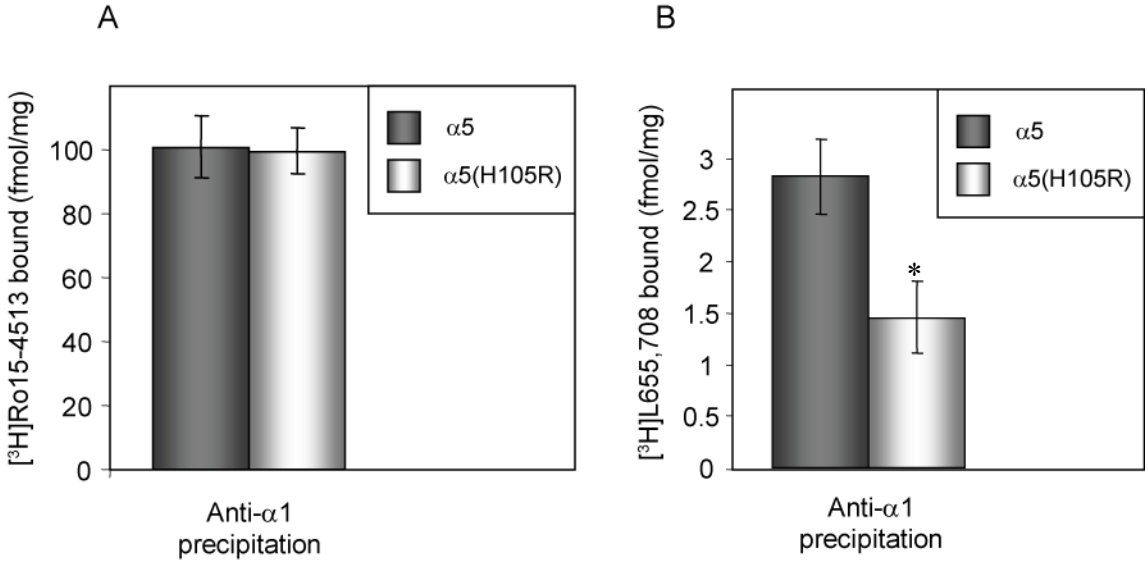

Figure 13. Reduced [^{3}H]L655,708 binding in GABA$_A$ receptors immunoprecipitated from α5(H105R) mouse brain membranes indicates altered α subunit positioning in α1α5(H105R) receptor complexes. Crude membranes prepared from whole mouse brains (5 mg protein/mg) were solubilized with 0.5 % deoxycholate and insoluble material removed by centrifugation (30 min, 100,000 x g). Aliquots of the resulting supernatant were subjected to immunoprecipitation with α1 antiserum. Antigen-antibody complexes were precipitated with Pansorbin and probed for radioligand binding. (A) [^{3}H]Ro15-4513 binding (12 nM) was unchanged in α1 immunoprecipitates of both wild type (101 ± 10 fmol/mg protein) and α5(H105R) mice (99 ± 7 fmol/mg protein). (B) Binding of the α5-selective radioligand [^{3}H]L655,708 (2 nM) to anti-α1 immunoprecipitates was 49 ± 6 % reduced in α5(H105R) mice (wild type 2.83 ± 0.36 fmol/mg protein, α5(H105R) mice 1.46 ± 0.35 fmol/mg protein; $P > 0.02$, Mann Whitney test). Nonspecific radioligand binding was determined by including excess clonazepam in parallel reactions. Results are given as mean ± SD of three independent experiments.

6.12 Potential involvement of α5-GABA$_A$ receptors in the modulation of NMDA receptor signaling cascades underlying learning and memory

The α5-containing GABA$_A$ receptors display a unique pattern of distribution in mammalian brain: they are abundantly expressed in the hippocampus where they are located mostly at the base of the spines and on the shafts of the dendrites (Fritschy *et al*. 1998, Brünig *et al*. 2002). This predominantly extrasynaptic location of α5-GABA$_A$ receptors suggests a potential modulation of NMDA receptor-mediated excitatory input at the spines (reviewed by Collingridge *et al*. 2004). The introduction of the H105R point mutation in the α5 subunit caused a reduction of α5-GABA$_A$ receptors in the hippocampus (Crestani *et al*. 2002) and resulted in a facilitated trace fear conditioning (Crestani *et al*. 2002), which is a hippocampus-dependent form of associative learning. This observation suggests that α5-GABA$_A$ receptors are contributing to the regulation of the dendritic excitability of hippocampal pyramidal cells and may be involved in controlling the NMDA receptor signaling cascade, known to play a role in learning and memory (Cammarota *et al*. 2000). To investigate whether the reduced hippocampal expression of α5-GABA$_A$ receptors affects downstream signaling events involved in learning and memory, we focused on key proteins of the NMDA receptor activated signaling cascade. We expected to pin down specific changes in the expression and/or activity of signaling proteins known to be part of NMDA receptor downstream signaling pathway involved in learning and memory: mitogen-activated protein kinases (p44/p42 MAPK), calcium/calmodulin-dependent protein kinase II (CaMKII) and cAMP response element-binding protein (CREB) (Roberson *et al*. 1996, Impey *et al*. 1998, Viola *et al*. 2000, West *et al*. 2002, Thomas and Huganir 2004, Leonard *et al*. 1999).

Proteins in downstream signaling pathways are often activated upon their phosphorylation at specific sites, which is therefore commonly used indicator of protein activity. In this study, hippocampal preparations from wild type and α5(H105R) knock-in mice were analyzed by Western blotting using pan- and phosphorylation site-specific antibodies. Experiments using two pools of four hippocampi of each genotype indicated a decreased phophorylation state of CREB, MAPK p44/p42 and CaMKII, with no change of their protein levels in α5(H105R) mice as compared to the wild type mice (Fig. 14A). Other protein kinases (PKCδ, PKCε, MAPK p38, SAPK/JNK, Src) tested in parallel, displayed no alterations of their phophorylation states (Fig. 14 B). The

reduction of phosphorylated CREB in α5(H105R) mice was further confirmed in three additional independent experiments using distinct pools of hippocampi. Therefore, in α5(H105R) mice, the phosphorylation levels, i.e. the activation states, of CREB, MAPK p44/p42 and CaMKII were selectively reduced.

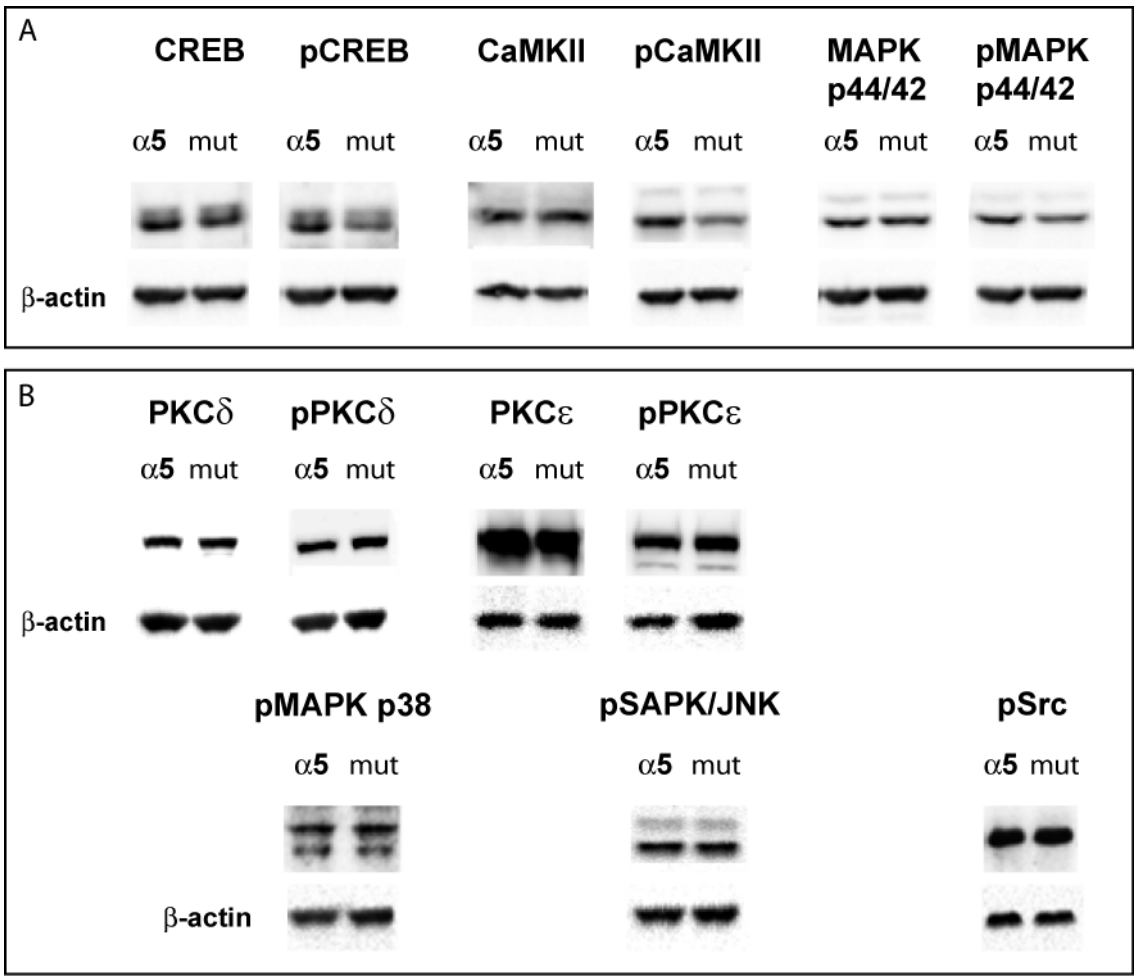

Figure 14. Altered activation levels of downstream-signaling proteins in α5(H105R) knock-in mice. Hippocampal proteins from wild type and α5(H105R) knock-in mice (pools of four animals) were analyzed by Western blotting using site-specific antibodies for total and phosphorylated, i. e. activated, forms of the indicated signaling proteins. (A) Unchanged expression levels, but reduced phophorylation of CREB, CaMKII and MAPK p44/42 in α5(H105R) knock-in mice. (B) Phosphorylation and expression levels of other kinases tested (PKCδ, PKCε, MAPK p38, SAPK/JNK, Src) were not affected. The levels of β-actin were tested in parallel on the same blots and used as a control for equal protein loading. The prefix 'p' indicates the phophorylated form of the respective signaling protein; wt, wild type mice; mut, α5(H105R) knock-in mice.

7 Discussion

$GABA_A$ receptors containing the α5 subunit are located mostly extrasynaptically, at the base of spines and on the adjacent shafts of dendrites of hippocampal pyramidal neurons (Brünig *et al*. 2002, Fritschy and Brünig 2003). They are mediators of a persistent low-amplitude tonic current (Caraiscos *et al*. 2004) and therefore expected to be involved in the modulation of dendritic excitability and the efficacy of excitatory inputs. The introduction of a point mutation (H105R) in the α5 subunit gene of mice caused a reduced expression of α5 subunit protein levels in the hippocampus, which resulted in enhanced trace fear conditioning (Crestani *et al*. 2002). These findings indicate that α5-$GABA_A$ receptors play an important role in the modulation of hippocampal learning and memory, possibly by modulating NMDA receptor mediated responses.

The present study aimed primarily at analyzing the consequences of the reduced levels of α5 subunit protein in α5(H105R) mice in respect to the assembled α5-$GABA_A$ receptor complex. In particular, we studied the influence of the α5(H105R) mutation on receptor expression, targeting, assembly and drug binding as well as on signaling proteins downstream of the NMDA receptor. There are two major results. 1) Receptor structure: The H105R mutation affects the assembly of functional, i. e. ligand binding α5(H105R)-$GABA_A$ receptors as well as the positioning of α subunits in α1α5-$GABA_A$ receptors and thereby the pharmacology of the benzodiazepine binding site. 2) Cellular signaling: The α5(H105R) mutation affects the activity of downstream signaling proteins involved in learning and memory.

7.1 Expression levels of α5 subunits in α5(H105R) mice

Previous studies on analogous α1(H101R), α2(H101R) and α3(H126R) knock-in mice demonstrated that introducing this point mutation did not affect the expression levels of the mutated subunit nor that of other major $GABA_A$ receptor subunits (Rudolph *et al*. 1999, Löw *et al*. 2000). Therefore, the moderate reduction of α5 subunit protein levels observed by Western blotting in whole brain tissue and by immunohistochemistry in the hippocampus of α5(H105R) mice by Crestani *et al*. (2002) was rather unexpected. Using membrane preparations from cortex/hippocampus of α5(H105R) mice a 23 ± 2 % reduction of α5(H105R) subunit protein levels was found. This finding corresponded

well to results published on the whole brain membrane preparations (20 ± 5 %, Crestani *et al*. 2002).

7.2 Targeting of α5-GABA$_A$ receptors

GABA$_A$ receptor assembly occurs within the endoplasmatic reticulum and it is crucial for efficient inhibitory neurotransmission that correctly assembled receptors are transported and inserted at the appropriate synaptic or extrasynaptic sites (Kittler *et al*. 2000, Moss and Smart 2001, Kittler *et al*. 2002). It was conceivable that the reduction of α5(H105R)-GABA$_A$ receptors might be a consequence of impaired receptor targeting.

However, transient expression of α5β3γ2 and α5(H105R)β3γ2 receptors in HEK293 cells resulted in similar expression levels and cell surface targeting of mutated and wild type receptors. The relative amount of plasma membrane-inserted α5 and α5(H105R) receptors amounted to about 60 % in both cases and indicated large intracellular pools of over-expressed subunits. The reason for similar expression levels and cell surface targeting of α5 and α5(H105R) receptors might be the over-expression of receptor subunits in the HEK293 cells, which may override subtle impairments. In addition, GABA$_A$ receptors are naturally expressed in neurons and thus might undergo different protein interactions, modifications and targeting than in HEK293 cells. Therefore, we analyzed targeting of α5(H105R)-GABA$_A$ receptors after transfection in neurons. To easily identify transfected neurons, wild type α5 subunits and α5(H105R) subunits were tagged with green fluorescent protein (GFP). GFP has been widely used as a tag to investigate the spatial and temporal organization of proteins in cells (reviewed by Lippincott-Schwartz and Patterson 2003). A key feature of this method is that GFP is genetically fused to the protein of interest and thus provides almost absolute detection specificity. In HEK293 cells, the GFP-tag did not affect expression or cell surface targeting of α5- and α5(H105R)-GABA$_A$ receptors. In neurons, both α5 and α5(H105R)-GFP-tagged subunits were highly expressed and targeted into proximal and distal neurits. This finding confirmed our previous results on comparable expression and targeting of α5 and α5(H105R) subunits in HEK293 cells. Therefore the H105R point mutation did not appear to influence the expression and targeting of α5 subunits upon over-expression in HEK293 cells or in cultured neurons. Thus, it is conceivable that subunit over-expression may compensate for minor disturbances that under normal

conditions would impair receptor expression, assembly or targeting. In this regard, results obtained using over-expressed receptors, even in neurons, should be interpreted with care.

7.3 Approaches to analyzing receptor trafficking

Methods to visualize and track $GABA_A$ receptors in living cells are central to characterize and understand their subcellular trafficking and targeting. Although fluorescent proteins have revolutionized such studies, they have shortcomings such as possible interference with the localization, structure or activity of the proteins to which they are fused (Lisenbee *et al*. 2003). In addition, puls-chase labeling techniques, which are required to analyze the trafficking and targeting of a given pool of proteins (e. g. trafficking of newly synthesized proteins to the cell surface) are not possible. Therefore, alternative approaches have been developed to label proteins in live cells (reviewed by Marks and Nolan 2006).

One very promising method that is ideally suited for pulse-chase experiments was developed by Tsien and colleagues: the biarsenical-tetracysteine labeling technique (Griffin *et al*. 1998). This labeling method is based on the introduction of a small tetracysteine tag (CCPGCC) into the protein of interest, which is recognized by membrane-permeable red (Lumio red) or green (Lumio green) fluorescent biarsenical dyes (reviewed by Zhang *et al.* 2002). This approach has been successfully applied to analyze the targeting of connexin 43 in HeLa cells (Gaietta *et al*. 2002) and AMPA receptors in neurons (Ju *et al*. 2004). Since this method enables pulse-chase experiments, we aimed at applying it to assess possible differences in α5- and α5(H105R)-$GABA_A$ receptor trafficking and targeting in neurons. Because the tetracysteine tag turned out to be difficult to insert into the α5 subunit, it was engineered into the α1 subunit (α1TetraCys) to test the suitability of this method for $GABA_A$ receptors. The tetracysteine tag was inserted into the cytoplasmic loop of the α1 subunit, since reducing conditions present in the cytoplasm are required for the covalent binding of the arsenic atoms of the dyes to the sulphur atoms of the tetracysteine motif. Although first labeling experiments with Lumio green and α1TetraCysβ2γ2 expressing HEK293 cells were quite promising, high background staining and toxicity of the dyes, particularly in neurons, prevented the application of this technique for studying $GABA_A$ receptor trafficking and targeting. Several attempts to reduce nonspecific staining as

well as toxicity of the dyes, including variation of the EDT_2 concentration, labeling and washing times failed. A major drawback of this method seems the significant affinity of biarsenical dyes for isolated thiols (Stroffekova *et al*. 2001). Thus, presumably lengthy and complex washouts are required to compete out arsenic monothiol interactions. However in many cases even extensive washing does not eliminate background staining (Stroffekova *et al*. 2001).

An alternative method that permits puls-chase experiments uses a recently introduced tag that represents the minimal binding site of the small polypeptide α-bungarotoxin derived from the muscle nicotinic acetylcholine receptor (Scherf *et al*. 2001). The neurotoxin α-bungarotoxin binds with high affinity and specificity to a 13 amino acid long sequence (WRYYESSLEPYPD), which can be introduced into any protein of interest to serve as a tag. α-Bungarotoxin is available in a fluorescent, biotinylated and radioactive form and has been successfully applied in a variety of assays including pulse-chase experiments (Scherf *et al*. 2001, Katchalski-Katzir *et al*. 2003). Since α-bungarotoxin is not able to permeate the plasma membrane, we placed the minimal binding sequence for α-bungarotoxin (btx) into the extracellular domain of the α5 subunit (α5btx). This strategy enables selective labeling of cell-surface receptors and analysis of membrane insertion and internalization of the receptor. The labeling procedure using AlexaFlour488-α-bungarotoxin was first established on HEK293 cells expressing the α5btxβ3γ2 subunit combination and yielded a staining pattern that perfectly colocalized with α5 and β3 antibody signals with virtually no background staining. This result is in accordance with similar studies on AMPA receptors (Sekine-Aizawa and Huganir 2004) and the β3 subunit of $GABA_A$ receptors (Bogdanov *et al*. 2006). Since we aimed at monitoring α5 and α5(H105R) receptors in neurons, we adapted this labeling strategy for cultured neurons transfected with the α5btx construct. One potential problem using neurons with this tagging method is the presence of endogenous α7 nicotinic acetylcholine receptors that binds α-bungarotoxin as well. Therefore, we blocked endogenous α-bungarotoxin binding sites with the antagonist tubocurarine as recommended by Sekine-Aizawa and Huganir (2004) prior to labeling of α5(btx)-$GABA_A$ receptors with AlexaFlour488-α-bungarotoxin. Unfortunately, nontransfected and transfected neurons unexpectedly showed significant background levels when labeled in this way with AlexaFlour488-α-bungarotoxin. This outcome

illustrated that the difficulty of blocking endogenous α-bungarotoxin binding sites in neurons is a major shortcoming of this method. Future experiments using higher concentrations of tubocurarine, or alternatively unlabeled α-bungarotoxin (Bogdanov *et al*. 2006), might, at least partially, reduce the background staining. For easier identification of transfected neurons it might be helpful to introduce an additional tag into the α5btx construct. However, our goal was to minimize number and size of tags to be introduced, because with every tag the risk to affect the physiological function and properties of the receptor complexes rises. This method clearly has the potential to trace important aspects in $GABA_A$ receptor trafficking.

7.4 The H105R mutation affects assembly of α5-$GABA_A$ receptors

Since the H105R mutation did not appreciably affect cell surface expression and targeting of α5 receptors into neurites, we hypothesized that the assembly of α5-$GABA_A$ receptors might be disturbed. This is a very likely hypothesis since it has been shown that amino acids involved in mediating inter-subunit contacts are in close proximity to histidine 105. To achieve the correct order of subunits around the pore, each subunit must be able to recognize and to interact with its neighbors via specific high-affinity contact sites. For some of the $GABA_A$ receptor subunits amino acid sequences that are important for the correct assembly of the receptor complex have been identified (α1: Taylor *et al*. 2000, Klausberger *et al*. 2000, Sarto *et al*. 2002b, Sarto-Jackson *et al*. 2006; β2/3: Taylor *et al*. 1999, Sarto *et al*. 2002b; γ2: Sarto *et al*. 2002b, Sarto-Jackson *et al*. 2006; γ3: Sarto *et al*. 2002a). In the case of association of α1 with γ2 subunits an important contact site is located proximal to the benzodiazepine binding site (Boilleau *et al*. 1999, Teissere and Czajkowski 2001). Most importantly, Sarto-Jackson *et al*. (2006) showed that one of the key amino acids for the assembly of the α1 subunit with β3 and γ2 subunits is alanine 108 (A108) in the α1 subunit. Thus, it is reasonable to assume that mutation of amino acids in close proximity to A108, like the H105R mutation in the α5 subunit, might affect assembly of the receptor complex.

For determination of the level of assembled $GABA_A$ receptors we made use of the fact that only fully assembled $GABA_A$ receptors containing a α, β and γ2 subunit bind benzodiazepine site ligands. Receptor autoradiography using [^{3}H]Ro15-4513, which binds to all $GABA_A$ receptor subtypes containing the γ2 subunit, revealed a 7 ± 0.5 % reduction of [^{3}H]Ro15-4513 binding in the hippocampus of α5(H105R) mice. Since

about 20% of $GABA_A$ receptors in the hippocampus contain the α5 subunit, i. e. display α5-$GABA_A$ receptor pharmacology (Sur *et al*. 1998), this level of reduction in [^{3}H]Ro15-4513 binding indicates a loss of about 30-40% of α5-$GABA_A$ receptors in the hippocampus of α5(H105R) mice. Since the level of α5 subunit protein was only reduced by about 20%, this finding indeed suggests that the H105R mutation might affect assembly of α5-$GABA_A$ receptors. In agreement with this observation, receptor autoradiography using the α5-selective ligand [^{3}H]L655,708 revealed a 47 ± 5 % reduction of binding in the whole hippocampus. In addition, the same level of reduction was observed in all other brain areas expressing α5-$GABA_A$ receptors, e. g. olfactory bulb (39 ± 4 %), superior colliculus (43 ± 0.4 %) and motor cortex (53 ± 6 %). Thus, the decrease of α5-$GABA_A$ receptors is not limited to the hippocampus as indicated by the published immunohistochemical data (Crestani *et al*. 2002), but concerns the entire α5-$GABA_A$ receptor population. Saturation binding experiments verified that the reduction in high affinity [^{3}H]L655,708 binding was not caused by a decreased affinity in response to the H105R mutation. The analysis demonstrated virtually identical K_D values for [^{3}H]L655,708 in brain membrane preparations from wild type and α5(H105R) mice but a strongly reduced number of binding sites (76 %) in α5(H105R) mice. These findings suggest the impairment of a fundamental process responsible for the receptor formation.

The discrepancy in levels of ligand binding to α5-$GABA_A$ receptors (76 % reduction) and expression levels of α5 subunit protein (23 % reduction) in α5(H105R) mice might be due to an inefficient assembly of the receptor complex, resulting in considerable amounts of unassembled α5 subunits. This hypothesis was tested by sucrose gradient centrifugation to separate high molecular assembled receptor complexes from unassembled low molecular size subunits. In brain extracts from α5(H105R) mice, the α1, α5, β2/3 and γ2 subunits were found exclusively in the high molecular fractions and thus indicated the presence of assembled α5(H105R)-$GABA_A$ receptors. However, [^{3}H]L655,708 binding was about 60% lower in the high molecular fractions derived from α5(H105R) mice than in fractions derived from control mice. This correlated well to the 76 % reduction of α5 receptor binding sites determined in the saturation binding experiments. Thus, although the binding of the α5-selective ligand [^{3}H]L655,708 was strongly reduced, the α5(H105R) subunits were found to assemble into macromolecular

complexes. This result suggests that a large proportion of α5-$GABA_A$ receptors assemble into severely impaired receptor complexes that are non-functional and do not bind ligands and are most likely destined for degradation. Degradation of misassembled receptor complexes is most likely mediated via the endoplasmatic reticulum-associated protein degradation pathway, which involves ubiquitination and translocation of the protein complexes from the ER into the cytoplasm for degradation by proteasomes (reviewed by Hirsch *et al.* 2004). This multi-step process may be slow and would lead to the intracellular accumulation of misassembled receptors, which would explain the moderately reduced expression levels of α5(H105R) subunit protein but a strong reduction of α5-selective ligand binding.

7.5 The H105R mutation affects positioning of α5 subunits within α1α5-$GABA_A$ receptors

It is interesting to note that immunodepletion experiments indicated that the His to Arg mutation solely rendered those $GABA_A$ receptors insensitive to diazepam binding, which contain two mutated α subunits in the receptor complex. Receptors containing one mutated and one non-mutated distinct type of α subunit, e. g. α1(H101R)α2-receptors, remained sensitive to diazepam binding (Benke *et al*. 2004). Mechanistically, the data implied that the non-mutated α subunit preferentially assembles with the γ2 subunit and thus conveys the respective benzodiazepine site binding properties to the receptor complex. In line with this finding, recent approaches linking the subunits covalently together, to assess the consequences of the subunit position within the receptor complex, demonstrated that only the α subunit next to the γ2 subunit determined the benzodiazepine binding characteristics of the receptor complex (Minier *et al*. 2004). Therefore, it might well be that the H105R mutation affects the positioning of the α5 subunit within receptors containing two different types of α subunits. Concerning α5-$GABA_A$ receptors, it has been found that a considerable proportion contain two distinct types of α subunits. In the α1-$GABA_A$ receptor population, which is the major $GABA_A$ receptor subtype (about 60 % of diazepam-sensitive $GABA_A$ receptors, Benke *et al*. 1991a, Benke *et al*. 1991b, Mertens *et al*. 1993), 9% of the receptors contain in addition to the α1 subunit the α5 subunit (Araujo *et al*. 1999). Furthermore, α2α5-$GABA_A$ receptors represent about 20 % of the α2-$GABA_A$ receptor population in the rat hippocampus (del Rio *et al*. 2001). Therefore, the fraction of

receptors containing in addition to the α5 subunit another type of α subunit may constitute about 50% of the pool of α5-$GABA_A$ receptors in the hippocampus. Interestingly, in both the α1α5-receptors and α2α5-receptors the α5 subunit was found to be pharmacologically dominant over the α1 subunit and α2 subunit, respectively, and solely displayed α5 pharmacology (Araujo *et al*. 1999, del Rio *et al*. 2001). Thus, in α5-$GABA_A$ receptors containing a second type of α subunit, e. g. α1 or α2, the α5 subunit would always be located next to the γ2 subunit.

To test the hypothesis that the H105R mutation might change the position of the α5 subunit, we estimated in a first set of experiments the relative abundance of the distinct zolpidem binding sites in wild type and mutant mice corresponding to the α1 (high affinity), α2/α3 (moderate affinity) and α5-$GABA_A$ receptor populations (low affinity) using zolpidem displacement of [^{3}H]Ro15-4513 binding. The rational behind this experimental approach was that if the H105R mutation decreases the affinity of the α5 subunit to associate with the γ2 subunit in a way that the second, non-mutated α subunit (e.g. α1) becomes the preferred partner of the γ2 subunit then the zolpidem binding properties of this receptor complex will be shifted from low affinity to high affinity. Thus, we expected to observe a shift in the relative abundance of low affinity zolpidem sites (α5) to high affinity zolpidem sites (α1). Indeed, in all individual experiments, we detected in preparations from α5(H105R) mice a slight increase in the relative abundance of high affinity zolpidem sites corresponding to α1-$GABA_A$ receptors and a concomitant decrease in low affinity zolpidem sites corresponding to α5-$GABA_A$ receptors. The decrease in low affinity zolpidem binding sites is, however, not only caused by a shift in the positioning of the α5 subunit in α1α5 receptor complexes but also due to the general down regulation of α5-$GABA_A$ receptors in the α5(H105R) mice. In addition, low expression levels of α4-$GABA_A$ receptors in hippocampus/cortex contribute to low affinity zolpidem sites (Benke *et al*. 1997) and is further compromising a quantitative interpretation of the abundance of low affinity zolpidem binding sites corresponding to α5-$GABA_A$ receptors. Nevertheless, the consistently observed small increase of high affinity zolpidem binding sites in α5(H105R) mice suggests that the H105R mutation may have influenced the positioning of the α5 subunit in receptor complexes containing in addition a α1 subunit.

Immunoprecipitation experiments provided further evidence that the positioning of the α5 subunit in α1α5-$GABA_A$ receptor might be changed in α5(H105R) mice. Using α1 subunit-selective antibodies the entire population of α1-$GABA_A$ receptors was isolated and the abundance α1α5-receptors was determined by high-affinity [^{3}H]L655,708 binding. The abundance of the total pool of α1 subunit containing $GABA_A$ receptors was similar in wild type and α5(H105R) mice as judged by [^{3}H]Ro15-4513 binding. However, high affinity [^{3}H]L655,708 binding, corresponding to α5 subunit properties, was reduced to about 50 % in α5(H105R) knock-in mice. Thus, this finding indicates that the α5(H105R) subunit was in about 50 % of cases pharmacologically inactive when assembled together with the α1 subunit in the receptor complex. Since in wild type animals the α5 subunit is pharmacologically dominant over the α1 subunit and therefore favorably arranged next to the γ2 subunit (Araujo *et al*. 1999), this finding indicates that the H105R mutation reduces the affinity of the α5 subunit to assemble with the γ2 subunit to a degree that the α1 subunit can take the position next to the γ2 subunit with the same probability as the α5(H105R) subunit.

7.6 Regulation by α5-$GABA_A$ receptors of downstream signaling proteins involved in hippocampal learning and memory

Genetic and pharmacological studies on α5-$GABA_A$ receptors in rodents indicated this subtype as one of the molecular substrates in hippocampal-dependent learning and memory processes (Collinson *et al*. 2002, Crestani *et al*. 2002, Chambers *et al*. 2003). The partial down regulation of α5-$GABA_A$ receptors in α5(H105R) mice resulted in an improved performance in the trace-fear conditioning paradigm (Crestani *et al*. 2002). It has been shown that memory consolidation on the cellular level is initiated by the activation of NMDA receptors, followed by changes in the level of second messengers, enhanced activity of protein kinases and transcription factors leading to *de novo* protein synthesis (Bozon *et al*. 2003, reviewed by Abel and Lattal 2001, reviewed by Nakazawa *et al*. 2004). Since the behavioral consequences of a reduced level of α5-$GABA_A$ receptors oppose those of a deficit of NMDA receptors (Tang *et al*. 1999, Shimizu *et al*. 2000), these two receptor systems appear to play a complementary role in controlling the signal transduction at hippocampal principal cells. Because α5-$GABA_A$ receptors are primarily found in extrasynaptic regions of spines and dendritic shafts of pyramidal

neurons (Crestani *et al*. 2002, Brünig *et al*. 2002), they are in a favored position to modulate excitatory input arising at the spines via NMDA receptors. If this is the case, activity of α5-$GABA_A$ receptors might be reflected in the modulation of the activity of signaling proteins involved in learning and memory activated by NMDA receptors.

It has been reported that in hippocampal principal cells α5-$GABA_A$ receptors are mediating tonic inhibition (Caraiscos *et al*. 2004, Scimemi *et al*. 2005, Prenosil *et al*. 2006) that is thought to play a critical role in regulation of network excitability (Semyanov *et al*. 2003) and information processing (Mitchell and Silver 2003). Although deletion or reduction of α5-$GABA_A$ receptors, respectively, improves certain aspects of cognitive performance (Collinson *et al*. 2002, Crestani *et al*. 2002) and suggests a negative influence of α5-$GABA_A$ receptors on cognition, they may be very important protecting neurons from excessive excitation by mediating tonic inhibition. Scimemi and colleagues (2005) showed that α5-$GABA_A$ receptors contribute to tonic inhibition virtually only under conditions of increased GABA concentrations. At lower GABA levels tonic inhibition appears to be mediated by δ-$GABA_A$ receptors. The δ-$GABA_A$ receptors are as well expressed extrasynaptically and display an exceptionally high affinity for GABA, which enables them to detect submicromolar concentrations of GABA (reviewed by Farrant and Nusser 2005). On the contrary, α5-$GABA_A$ receptors are activated by higher GABA concentrations, which are generated as a result of enhanced physiological activities (Bianchi *et al*. 2003) and pathological states (Smolders *et al*. 2004). Thus, α5-$GABA_A$ receptors come into play during periods of activity, which perfectly correlates with the activity of NMDA receptors and therefore are very well suited to modulate their excitatory input.

Since NMDA receptors control the CaMKII and MAPK pathways, which both converge at the level of the transcription factor CREB (Wu *et al*. 2001, reviewed by West *et al*. 2002), we analyzed the activation states of the key proteins of these signaling cascades in hippocampi of α5(H105R) mice as compared to wild type mice. Interestingly, we detected reduced phosphorylation levels, i. e. activation states, selectively for CaMKII, p44/42 MAPK and CREB in the hippocampus of α5(H105R) mice with no change in their protein levels. This effect was specific since the activation states of several other protein kinases tested in parallel remained unchanged. At present, it is not clear why the activity of CaMKII, p44/42 MAPK and CREB is reduced in α5(H105R) mice. A

reduced expression of functional α5-$GABA_A$ receptors in the hippocampus is expected to result in a reduced inhibitory tone at spines of hippocampal pyramidal neurons and thus to an increase in NMDA receptor activity. An increased activity of synaptic NMDA receptors would consequently result in an enhanced activity of downstream signaling proteins. However it may well be that the activity of CaMKII, p44/42 MAPK and CREB is down-regulated to compensate for a chronically enhanced NMDA receptor response.

Activated CREB, i. e. CREB phosphorylated at Ser133 (pCREB), has been shown to be time-dependently increased in inhibitory avoidance training and spatial novelty tests (Impey *et al*. 1998, Taubenfeld *et al*. 1999, Cammarota *et al*. 2000, Viola *et al*. 2000, Vianna *et al*. 2000). In future experiments, it would be interesting to analyze how CREB activity is regulated in α5(H105) mice subjected to these tests.

8 Conclusions

The data of the current study indicate that the H105R mutation in the α5 subunit severely affects the assembly of functional, i. e. ligand binding α5-$GABA_A$ receptors. A large fraction (~50%) of mutated α5-$GABA_A$ receptors form high molecular complexes similar to non-mutated α5-$GABA_A$ receptors but do not bind ligands of the benzodiazepine binding site. They most likely represent misassembled, non-functional receptors to be degraded. Since degradation by the endoplasmatic reticulum-associated protein degradation pathway is a relatively slow process (reviewed by Hirsch *et al.* 2004) this may be reflected by the moderately reduced (~20%) expression levels of α5(H105R) subunit protein.

In addition, α5(H105R) subunits that successfully assemble into functional receptors appear to display a reduced affinity to associate with the γ2 subunit. This leads to a further reduction of α5-specific drug binding properties in receptors containing in addition to the mutated α5 subunit the α1 subunit (α1α5-$GABA_A$ receptors).

The strong reduction of functional α5-$GABA_A$ receptors in α5(H105R) mice is expected to result in an reduced inhibitory tone at hippocampal pyramidal spines, leading to enhanced NMDA receptor activity. The reduced activity of downstream signaling proteins (CaMKII, MAPK 44/42 and CREB) observed in α5(H105R) mice might therefore reflect an adaptive response to compensate for the enhanced NMDA receptor signaling.

9 References

Abel T, Lattal KM (2001) Molecular mechanisms of memory acquisition, consolidation and retrieval. Curr Opin Neurobiol 11:180-187.

Adams SR, Campbell RE, Gross LA, Martin BR, Walkup GK, Yao Y, Llopis J, Tsien RY (2002) New biarsenical ligands and tetracysteine motifs for protein labeling in vitro and in vivo: synthesis and biological applications. J Am Chem Soc 124:6063-6076.

Araujo F, Ruano D, Vitorica J (1999) Native gamma-aminobutyric acid type A receptors from rat hippocampus, containing both α1 and α5 subunits, exhibit a single benzodiazepine binding site with α5 pharmacological properties. J Pharmacol Exp Ther 290:989-997.

Atack JR, Wafford KA, Tye SJ, Cook SM, Sohal B, Pike A, Sur C, Melillo D, Bristow L, Bromidge F, Ragan I, Kerby J, Street L, Carling R, Castro JL, Whiting P, Dawson GR, McKernan RM (2006) TPA023 [7-(1,1-dimethylethyl)-6-(2-ethyl-2H-1,2,4-triazol-3-methoxy)-3-(2-fluorophenyl)-1,2,4-triazolo[4,3-b]-pyridazine], an agonist selective for α2- and α3-containing $GABA_A$ receptors, is a nonsedating anxiolytic in rodents and primates. J Pharmacol Exp Ther 316:410-422.

Athos J, Impey S, Pineda VV, Chen X, Storm DR (2002) Hippocampal CRE-mediated gene expression is required for contextual memory formation. Nat Neurosci 5:1119-1120.

Atkins CM, Selcher JC, Petraitis JJ, Trzaskos JM, Sweatt JD (1998) The MAPK cascade is required for mammalian associative learning. Nat Neurosci 1:602-609.

Barnard EA, Skolnick P, Olsen RW, Mohler H, Sieghart W, Biggio G, Braestrup C, Bateson AN, Langer SZ (1998) International Union of Pharmacology. XV. Subtypes of gamma-aminobutyric acid A receptors: classification on the basis of subunit structure and receptor function. Pharmacol Rev 50:291-313.

Baur R, Sigel E (2005) Influence of the point mutation α1-H101R on the assembly of gamma-aminobutyric acid type A receptors. Neuroreport 16:1955-1958.

Benavides J, Peny B, Ruano D, Vitorica J, Scatton B (1993) Comparative autoradiographic distribution of central omega (benzodiazepine) modulatory site subtypes with high, intermediate and low affinity for zolpidem and alpidem.

Brain Res 604:240-250.

Benke D, Cicin-Sain A, Mertens S, Mohler H (1991a) Immunochemical identification of the α1- and α3-subunits of the $GABA_A$-receptor in rat brain. J Recept Res 11:407-424.

Benke D, Fakitsas P, Roggenmoser C, Michel C, Rudolph U, Mohler H (2004) Analysis of the presence and abundance of $GABA_A$ receptors containing two different types of α subunits in murine brain using point-mutated α subunits. J Biol Chem 279:43654-43660.

Benke D, Honer M, Michel C, Mohler H (1996) $GABA_A$ receptor subtypes differentiated by their γ-subunit variants: prevalence, pharmacology and subunit architecture. Neuropharmacology 35:1413-1423.

Benke D, Mertens S, Trzeciak A, Gillessen D, Mohler H (1991b) $GABA_A$ receptors display association of γ2-subunit with α1- and β2/3-subunits. J Biol Chem 266:4478-4483.

Benke D, Michel C, Mohler H (1997) $GABA_A$ receptors containing the α4-subunit: prevalence, distribution, pharmacology, and subunit architecture in situ. J Neurochem 69:806-814.

Benson JA, Low K, Keist R, Mohler H, Rudolph U (1998) Pharmacology of recombinant gamma-aminobutyric acid A receptors rendered diazepam-insensitive by point-mutated α-subunits. FEBS Lett 431:400-404.

Bianchi L, Ballini C, Colivicchi MA, Della Corte L, Giovannini MG, Pepeu G (2003) Investigation on acetylcholine, aspartate, glutamate and GABA extracellular levels from ventral hippocampus during repeated exploratory activity in the rat. Neurochem Res 28:565-573.

Bogdanov Y, Michels G, Armstrong-Gold C, Haydon PG, Lindstrom J, Pangalos M, Moss SJ (2006) Synaptic $GABA_A$ receptors are directly recruited from their extrasynaptic counterparts. Embo J 25:4381-4389.

Boileau AJ, Evers AR, Davis AF, Czajkowski C (1999) Mapping the agonist binding site of the $GABA_A$ receptor: evidence for a β-strand. J Neurosci 19:4847-4854.

Bollan K, King D, Robertson LA, Brown K, Taylor PM, Moss SJ, Connolly CN (2003) $GABA_A$ receptor composition is determined by distinct assembly signals within α and β subunits. J Biol Chem 278:4747-4755.

Bollan K, Robertson LA, Tang H, Connolly CN (2003) Multiple assembly signals in

gamma-aminobutyric acid (type A) receptor subunits combine to drive receptor construction and composition. Biochem Soc Trans 31:875-879.

Borden LA (1996) GABA transporter heterogeneity: pharmacology and cellular localization. Neurochem Int 29:335-356.

Bormann J (2000) The 'ABC' of GABA receptors. Trends Pharmacol Sci 21:16-19.

Bourtchuladze R, Frenguelli B, Blendy J, Cioffi D, Schutz G, Silva AJ (1994) Deficient long-term memory in mice with a targeted mutation of the cAMP-responsive element-binding protein. Cell 79:59-68.

Bozon B, Kelly A, Josselyn SA, Silva AJ, Davis S, Laroche S (2003) MAPK, CREB and zif268 are all required for the consolidation of recognition memory. Philos Trans R Soc Lond B Biol Sci 358:805-814.

Brewer GJ, Cotman CW (1989) Survival and growth of hippocampal neurons in defined medium at low density: advantages of a sandwich culture technique or low oxygen. Brain Res 494:65-74.

Broadbent NJ, Squire LR, Clark RE (2004) Spatial memory, recognition memory, and the hippocampus. Proc Natl Acad Sci U S A 101:14515-14520.

Brunig I, Scotti E, Sidler C, Fritschy JM (2002) Intact sorting, targeting, and clustering of gamma-aminobutyric acid A receptor subtypes in hippocampal neurons in vitro. J Comp Neurol 443:43-55.

Cammarota M, Bevilaqua LR, Ardenghi P, Paratcha G, Levi de Stein M, Izquierdo I, Medina JH (2000) Learning-associated activation of nuclear MAPK, CREB and Elk-1, along with Fos production, in the rat hippocampus after a one-trial avoidance learning: abolition by NMDA receptor blockade. Brain Res Mol Brain Res 76:36-46.

Caraiscos VB, Elliott EM, You-Ten KE, Cheng VY, Belelli D, Newell JG, Jackson MF, Lambert JJ, Rosahl TW, Wafford KA, MacDonald JF, Orser BA (2004) Tonic inhibition in mouse hippocampal CA1 pyramidal neurons is mediated by α5 subunit-containing gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101:3662-3667.

Casula MA, Bromidge FA, Pillai GV, Wingrove PB, Martin K, Maubach K, Seabrook GR, Whiting PJ, Hadingham KL (2001) Identification of amino acid residues responsible for the α5 subunit binding selectivity of L-655,708, a benzodiazepine binding site ligand at the $GABA_A$ receptor. J Neurochem

77:445-451.

Chambers MS, Atack JR, Broughton HB, Collinson N, Cook S, Dawson GR, Hobbs SC, Marshall G, Maubach KA, Pillai GV, Reeve AJ, MacLeod AM (2003) Identification of a novel, selective $GABA_A$ α5 receptor inverse agonist which enhances cognition. J Med Chem 46:2227-2240.

Chambers MS, Atack JR, Carling RW, Collinson N, Cook SM, Dawson GR, Ferris P, Hobbs SC, O'Connor D, Marshall G, Rycroft W, Macleod AM (2004) An orally bioavailable, functionally selective inverse agonist at the benzodiazepine site of $GABA_A$ α5 receptors with cognition enhancing properties. J Med Chem 47:5829-5832.

Chen C, Okayama H (1987) High-efficiency transformation of mammalian cells by plasmid DNA. Mol Cell Biol 7:2745-2752.

Chen I, Ting AY (2005) Site-specific labeling of proteins with small molecules in live cells. Curr Opin Biotechnol 16:35-40.

Chen ZW, Olsen RW (2007) $GABA_A$ receptor associated proteins: a key factor regulating $GABA_A$ receptor function. J Neurochem 100:279-294.

Chiara DC, Cohen JB (1997) Identification of amino acids contributing to high and low affinity d-tubocurarine sites in the Torpedo nicotinic acetylcholine receptor. J Biol Chem 272:32940-32950.

Collingridge GL, Isaac JT, Wang YT (2004) Receptor trafficking and synaptic plasticity. Nat Rev Neurosci 5:952-962.

Collinson N, Kuenzi FM, Jarolimek W, Maubach KA, Cothliff R, Sur C, Smith A, Otu FM, Howell O, Atack JR, McKernan RM, Seabrook GR, Dawson GR, Whiting PJ, Rosahl TW (2002) Enhanced learning and memory and altered GABAergic synaptic transmission in mice lacking the α5 subunit of the $GABA_A$ receptor. J Neurosci 22:5572-5580.

Connolly CN, Krishek BJ, McDonald BJ, Smart TG, Moss SJ (1996) Assembly and cell surface expression of heteromeric and homomeric gamma-aminobutyric acid type A receptors. J Biol Chem 271:89-96.

Connolly CN, Wafford KA (2004) The Cys-loop superfamily of ligand-gated ion channels: the impact of receptor structure on function. Biochem Soc Trans 32:529-534.

Crestani F, Keist R, Fritschy JM, Benke D, Vogt K, Prut L, Bluthmann H, Mohler H,

Rudolph U (2002) Trace fear conditioning involves hippocampal α5 $GABA_A$ receptors. Proc Natl Acad Sci U S A 99:8980-8985.

Crestani F, Lorez M, Baer K, Essrich C, Benke D, Laurent JP, Belzung C, Fritschy JM, Luscher B, Mohler H (1999) Decreased $GABA_A$-receptor clustering results in enhanced anxiety and a bias for threat cues. Nat Neurosci 2:833-839.

Deisseroth K, Bito H, Tsien RW (1996) Signaling from synapse to nucleus: postsynaptic CREB phosphorylation during multiple forms of hippocampal synaptic plasticity. Neuron 16:89-101.

del Rio JC, Araujo F, Ramos B, Ruano D, Vitorica J (2001) Prevalence between different α subunits performing the benzodiazepine binding sites in native heterologous $GABA_A$ receptors containing the α2 subunit. J Neurochem 79:183-191.

DeLorey TM, Handforth A, Anagnostaras SG, Homanics GE, Minassian BA, Asatourian A, Fanselow MS, Delgado-Escueta A, Ellison GD, Olsen RW (1998) Mice lacking the β3 subunit of the $GABA_A$ receptor have the epilepsy phenotype and many of the behavioral characteristics of Angelman syndrome. J Neurosci 18:8505-8514.

Dilger JP (2002) The effects of general anaesthetics on ligand-gated ion channels. Br J Anaesth 89:41-51.

Dumoulin A, Rostaing P, Bedet C, Levi S, Isambert MF, Henry JP, Triller A, Gasnier B (1999) Presence of the vesicular inhibitory amino acid transporter in GABAergic and glycinergic synaptic terminal boutons. J Cell Sci 112 (Pt 6):811-823.

Ernst M, Bruckner S, Boresch S, Sieghart W (2005) Comparative models of $GABA_A$ receptor extracellular and transmembrane domains: important insights in pharmacology and function. Mol Pharmacol 68:1291-1300.

Farrant M, Nusser Z (2005) Variations on an inhibitory theme: phasic and tonic activation of $GABA_A$ receptors. Nat Rev Neurosci 6:215-229.

Fritschy JM, Benke D, Johnson DK, Mohler H, Rudolph U (1997) $GABA_A$ receptor α-subunit is an essential prerequisite for receptor formation in vivo. Neuroscience 81:1043-1053.

Fritschy JM, Brunig I (2003) Formation and plasticity of GABAergic synapses: physiological mechanisms and pathophysiological implications. Pharmacol Ther 98:299-323.

Fritschy JM, Mohler H (1995) $GABA_A$-receptor heterogeneity in the adult rat brain: differential regional and cellular distribution of seven major subunits. J Comp Neurol 359:154-194.

Fritschy JM, Weinmann O, Wenzel A, Benke D (1998) Synapse-specific localization of NMDA and $GABA_A$ receptor subunits revealed by antigen-retrieval immunohistochemistry. J Comp Neurol 390:194-210.

Gaietta G, Deerinck TJ, Adams SR, Bouwer J, Tour O, Laird DW, Sosinsky GE, Tsien RY, Ellisman MH (2002) Multicolor and electron microscopic imaging of connexin trafficking. Science 296:503-507.

Glykys J, Mody I (2006) Hippocampal network hyperactivity after selective reduction of tonic inhibition in $GABA_A$ receptor $\alpha5$ subunit-deficient mice. J Neurophysiol 95:2796-2807.

Gorrie GH, Vallis Y, Stephenson A, Whitfield J, Browning B, Smart TG, Moss SJ (1997) Assembly of $GABA_A$ receptors composed of $\alpha1$ and $\beta2$ subunits in both cultured neurons and fibroblasts. J Neurosci 17:6587-6596.

Griffin A, Adams, S. R., Jones, J., Tsien, R. Y. (2000) Fluorescent labeling of recombinant proteins in living cells with FlAsH. Methods Enzymol 327:565-578.

Griffin BA, Adams SR, Tsien RY (1998) Specific covalent labeling of recombinant protein molecules inside live cells. Science 281:269-272.

Grover LM, Yan C (1999) Blockade of $GABA_A$ receptors facilitates induction of NMDA receptor-independent long-term potentiation. J Neurophysiol 81:2814-2822.

Hardingham GE, Fukunaga Y, Bading H (2002) Extrasynaptic NMDARs oppose synaptic NMDARs by triggering CREB shut-off and cell death pathways. Nat Neurosci 5:405-414.

Harel M, Kasher R, Nicolas A, Guss JM, Balass M, Fridkin M, Smit AB, Brejc K, Sixma TK, Katchalski-Katzir E, Sussman JL, Fuchs S (2001) The binding site of acetylcholine receptor as visualized in the X-Ray structure of a complex between α-bungarotoxin and a mimotope peptide. Neuron 32:265-275.

Hauser J, Rudolph U, Keist R, Mohler H, Feldon J, Yee BK (2005) Hippocampal $\alpha5$ subunit-containing $GABA_A$ receptors modulate the expression of prepulse inhibition. Mol Psychiatry 10:201-207.

Hirsch C, Jarosch E, Sommer T, Wolf DH (2004) Endoplasmic reticulum-associated protein degradation - one model fits all? Biochim Biophys Acta 1695:215-223.

Huang Q, He X, Ma C, Liu R, Yu S, Dayer CA, Wenger GR, McKernan R, Cook JM (2000) Pharmacophore/receptor models for $GABA_A$/BzR subtypes ($\alpha1\beta3\gamma2$, $\alpha5\beta3\gamma2$, and $\alpha6\beta3\gamma2$) via a comprehensive ligand-mapping approach. J Med Chem 43:71-95.

Huerta PT, Sun LD, Wilson MA, Tonegawa S (2000) Formation of temporal memory requires NMDA receptors within CA1 pyramidal neurons. Neuron 25:473-480.

Impey S, Smith DM, Obrietan K, Donahue R, Wade C, Storm DR (1998) Stimulation of cAMP response element (CRE)-mediated transcription during contextual learning. Nat Neurosci 1:595-601.

Izquierdo I, Schroder N, Netto CA, Medina JH (1999) Novelty causes time-dependent retrograde amnesia for one-trial avoidance in rats through NMDA receptor- and CaMKII-dependent mechanisms in the hippocampus. Eur J Neurosci 11:3323-3328.

Job C, Eberwine J (2001) Localization and translation of mRNA in dendrites and axons. Nat Rev Neurosci 2:889-898.

Ju W, Morishita W, Tsui J, Gaietta G, Deerinck TJ, Adams SR, Garner CC, Tsien RY, Ellisman MH, Malenka RC (2004) Activity-dependent regulation of dendritic synthesis and trafficking of AMPA receptors. Nat Neurosci 7:244-253.

Kash TL, Trudell JR, Harrison NL (2004) Structural elements involved in activation of the gamma-aminobutyric acid type A ($GABA_A$) receptor. Biochem Soc Trans 32:540-546.

Katchalski-Katzir E, Kasher R, Balass M, Scherf T, Harel M, Fridkin M, Sussman JL, Fuchs S (2003) Design and synthesis of peptides that bind α-bungarotoxin with high affinity and mimic the three-dimensional structure of the binding-site of acetylcholine receptor. Biophys Chem 100:293-305.

Kelly MD, Smith A, Banks G, Wingrove P, Whiting PW, Atack J, Seabrook GR, Maubach KA (2002) Role of the histidine residue at position 105 in the human $\alpha5$ containing $GABA_A$ receptor on the affinity and efficacy of benzodiazepine site ligands. Br J Pharmacol 135:248-256.

Kittler JT, Delmas P, Jovanovic JN, Brown DA, Smart TG, Moss SJ (2000) Constitutive endocytosis of $GABA_A$ receptors by an association with the adaptin

AP2 complex modulates inhibitory synaptic currents in hippocampal neurons. J Neurosci 20:7972-7977.

Kittler JT, McAinsh K, Moss SJ (2002) Mechanisms of $GABA_A$ receptor assembly and trafficking: implications for the modulation of inhibitory neurotransmission. Mol Neurobiol 26:251-268.

Kittler JT, Moss SJ (2003) Modulation of $GABA_A$ receptor activity by phosphorylation and receptor trafficking: implications for the efficacy of synaptic inhibition. Curr Opin Neurobiol 13:341-347.

Kittler JT, Wang J, Connolly CN, Vicini S, Smart TG, Moss SJ (2000) Analysis of $GABA_A$ receptor assembly in mammalian cell lines and hippocampal neurons using $\gamma 2$ subunit green fluorescent protein chimeras. Mol Cell Neurosci 16:440-452.

Klausberger T, Fuchs K, Mayer B, Ehya N, Sieghart W (2000) $GABA_A$ receptor assembly. Identification and structure of $\gamma 2$ sequences forming the intersubunit contacts with $\alpha 1$ and $\beta 3$ subunits. J Biol Chem 275:8921-8928.

Kunig G, Leenders KL, Sanchez-Pernaute R, Antonini A, Vontobel P, Verhagen A, Gunther I (2000) Benzodiazepine receptor binding in Huntington's disease: [^{11}C]flumazenil uptake measured using positron emission tomography. Ann Neurol 47:644-648.

Laemmli UK (1970) Cleavage of structural proteins during the assembly of the head of bacteriophage T4. Nature 227:680-685.

Leonard AS, Lim IA, Hemsworth DE, Horne MC, Hell JW (1999) Calcium/calmodulin-dependent protein kinase II is associated with the N-methyl-D-aspartate receptor. Proc Natl Acad Sci U S A 96:3239-3244.

Li M, Szabo A, Rosenberg HC (2001) Evaluation of native $GABA_A$ receptors containing an $\alpha 5$ subunit. Eur J Pharmacol 413:63-72.

Lippincott-Schwartz J, Patterson GH (2003) Development and use of fluorescent protein markers in living cells. Science 300:87-91.

Lisenbee CS, Karnik SK, Trelease RN (2003) Overexpression and mislocalization of a tail-anchored GFP redefines the identity of peroxisomal ER. Traffic 4:491-501.

Loebrich S, Bahring R, Katsuno T, Tsukita S, Kneussel M (2006) Activated radixin is essential for $GABA_A$ receptor $\alpha 5$ subunit anchoring at the actin cytoskeleton. Embo J 25:987-999.

Lonze BE, Ginty DD (2002) Function and regulation of CREB family transcription factors in the nervous system. Neuron 35:605-623.

Low K, Crestani F, Keist R, Benke D, Brunig I, Benson JA, Fritschy JM, Rulicke T, Bluethmann H, Mohler H, Rudolph U (2000) Molecular and neuronal substrate for the selective attenuation of anxiety. Science 290:131-134.

Luscher B, Keller CA (2004) Regulation of $GABA_A$ receptor trafficking, channel activity, and functional plasticity of inhibitory synapses. Pharmacol Ther 102:195-221.

Macdonald RL, Olsen RW (1994) $GABA_A$ receptor channels. Annu Rev Neurosci 17:569-602.

Malizia AL (1999) What do brain imaging studies tell us about anxiety disorders? J Psychopharmacol 13:372-378.

Marks KM, Nolan GP (2006) Chemical labeling strategies for cell biology. Nat Methods 3:591-596.

Marksitzer R, Benke D, Fritschy JM, Trzeciak A, Bannwarth W, Mohler H (1993) $GABA_A$-receptors: drug binding profile and distribution of receptors containing the α2-subunit in situ. J Recept Res 13:467-477.

Mayr B, Montminy M (2001) Transcriptional regulation by the phosphorylation-dependent factor CREB. Nat Rev Mol Cell Biol 2:599-609.

McCann CM, Bareyre FM, Lichtman JW, Sanes JR (2005) Peptide tags for labeling membrane proteins in live cells with multiple fluorophores. Biotechniques 38:945-952.

McGaugh JL (2000) Memory - a century of consolidation. Science 287:248-251.

McKernan RM, Rosahl TW, Reynolds DS, Sur C, Wafford KA, Atack JR, Farrar S, Myers J, Cook G, Ferris P, Garrett L, Bristow L, Marshall G, Macaulay A, Brown N, Howell O, Moore KW, Carling RW, Street LJ, Castro JL, Ragan CI, Dawson GR, Whiting PJ (2000) Sedative but not anxiolytic properties of benzodiazepines are mediated by the $GABA_A$ receptor α1 subtype. Nat Neurosci 3:587-592.

McKernan RM, Whiting PJ (1996) Which $GABA_A$-receptor subtypes really occur in the brain? Trends Neurosci 19:139-143.

Mertens S, Benke D, Mohler H (1993) $GABA_A$ receptor populations with novel subunit combinations and drug binding profiles identified in brain by α5- and δ-subunit-

specific immunopurification. J Biol Chem 268:5965-5973.

Minier F, Sigel E (2004) Positioning of the α-subunit isoforms confers a functional signature to gamma-aminobutyric acid type A receptors. Proc Natl Acad Sci U S A 101:7769-7774.

Mitchell SJ, Silver RA (2003) Shunting inhibition modulates neuronal gain during synaptic excitation. Neuron 38:433-445.

Mohler H (2006) $GABA_A$ receptor diversity and pharmacology. Cell Tissue Res 326:505-516.

Mohler H (2007) Molecular regulation of cognitive functions and developmental plasticity: impact of $GABA_A$ receptors. J Neurochem 102:1-12.

Mohler H, Fritschy JM, Rudolph U (2002) A new benzodiazepine pharmacology. J Pharmacol Exp Ther 300:2-8.

Montminy M (1997) Transcriptional regulation by cyclic AMP. Annu Rev Biochem 66:807-822.

Morrow AL, VanDoren MJ, Penland SN, Matthews DB (2001) The role of GABAergic neuroactive steroids in ethanol action, tolerance and dependence. Brain Res Brain Res Rev 37:98-109.

Moss SJ, Smart TG (2001) Constructing inhibitory synapses. Nat Rev Neurosci 2:240-250.

Nakazawa K, McHugh TJ, Wilson MA, Tonegawa S (2004) NMDA receptors, place cells and hippocampal spatial memory. Nat Rev Neurosci 5:361-372.

Nutt DJ, Malizia AL (2001) New insights into the role of the $GABA_A$-benzodiazepine receptor in psychiatric disorder. Br J Psychiatry 179:390-396.

Olsen RW, DeLorey TM, Gordey M, Kang MH (1999) GABA receptor function and epilepsy. Adv Neurol 79:499-510.

Owens DF, Kriegstein AR (2002) Is there more to GABA than synaptic inhibition? Nat Rev Neurosci 3:715-727.

Prenosil GA, Schneider Gasser EM, Rudolph U, Keist R, Fritschy JM, Vogt KE (2006) Specific subtypes of $GABA_A$ receptors mediate phasic and tonic forms of inhibition in hippocampal pyramidal neurons. J Neurophysiol 96:846-857.

Quick MW, Corey JL, Davidson N, Lester HA (1997) Second messengers, trafficking-related proteins, and amino acid residues that contribute to the functional regulation of the rat brain GABA transporter GAT1. J Neurosci 17:2967-2979.

Quirk K, Blurton P, Fletcher S, Leeson P, Tang F, Mellilo D, Ragan CI, McKernan RM (1996) [^{3}H]L-655,708, a novel ligand selective for the benzodiazepine site of $GABA_A$ receptors which contain the α5 subunit. Neuropharmacology 35:1331-1335.

Roberson ED, English JD, Adams JP, Selcher JC, Kondratick C, Sweatt JD (1999) The mitogen-activated protein kinase cascade couples PKA and PKC to cAMP response element binding protein phosphorylation in area CA1 of hippocampus. J Neurosci 19:4337-4348.

Rodriguez Manzanares PA, Isoardi NA, Carrer HF, Molina VA (2005) Previous stress facilitates fear memory, attenuates GABAergic inhibition, and increases synaptic plasticity in the rat basolateral amygdala. J Neurosci 25:8725-8734.

Ruano D, Vizuete M, Cano J, Machado A, Vitorica J (1992) Heterogeneity in the allosteric interaction between the gamma-aminobutyric acid (GABA) binding site and three different benzodiazepine binding sites of the $GABA_A$/benzodiazepine receptor complex in the rat nervous system. J Neurochem 58:485-493.

Rudner L, Nydegger S, Coren LV, Nagashima K, Thali M, Ott DE (2005) Dynamic fluorescent imaging of human immunodeficiency virus type 1 gag in live cells by biarsenical labeling. J Virol 79:4055-4065.

Rudolph U, Crestani F, Benke D, Brunig I, Benson JA, Fritschy JM, Martin JR, Bluethmann H, Mohler H (1999) Benzodiazepine actions mediated by specific gamma-aminobutyric acid A receptor subtypes. Nature 401:796-800.

Rudolph U, Mohler H (2004) Analysis of $GABA_A$ receptor function and dissection of the pharmacology of benzodiazepines and general anesthetics through mouse genetics. Annu Rev Pharmacol Toxicol 44:475-498.

Rudolph U, Mohler H (2006) GABA-based therapeutic approaches: $GABA_A$ receptor subtype functions. Curr Opin Pharmacol 6:18-23.

Sarto I, Klausberger T, Ehya N, Mayer B, Fuchs K, Sieghart W (2002a) A novel site on γ3 subunits important for assembly of $GABA_A$ receptors. J Biol Chem 277:30656-30664.

Sarto I, Wabnegger L, Dogl E, Sieghart W (2002b) Homologous sites of $GABA_A$ receptor α1, β3 and γ2 subunits are important for assembly. Neuropharmacology 43:482-491.

Sarto-Jackson I, Ramerstorfer J, Ernst M, Sieghart W (2006) Identification of amino acid residues important for assembly of GABA receptor α1 and γ2 subunits. J Neurochem 96:983-995.

Scherf T, Kasher R, Balass M, Fridkin M, Fuchs S, Katchalski-Katzir E (2001) A β-hairpin structure in a 13-mer peptide that binds α-bungarotoxin with high affinity and neutralizes its toxicity. Proc Natl Acad Sci U S A 98:6629-6634.

Schoch P, Richards JG, Haring P, Takacs B, Stahli C, Staehelin T, Haefely W, Mohler H (1985) Co-localization of GABA receptors and benzodiazepine receptors in the brain shown by monoclonal antibodies. Nature 314:168-171.

Schousboe A, Waagepetersen HS (2007) GABA: Homeostatic and pharmacological aspects. Prog Brain Res 160:9-19.

Scimemi A, Semyanov A, Sperk G, Kullmann DM, Walker MC (2005) Multiple and plastic receptors mediate tonic $GABA_A$ receptor currents in the hippocampus. J Neurosci 25:10016-10024.

Sekine-Aizawa Y, Huganir RL (2004) Imaging of receptor trafficking by using α-bungarotoxin-binding-site-tagged receptors. Proc Natl Acad Sci U S A 101:17114-17119.

Semyanov A, Walker MC, Kullmann DM (2003) GABA uptake regulates cortical excitability via cell type-specific tonic inhibition. Nat Neurosci 6:484-490.

Serwanski DR, Miralles CP, Christie SB, Mehta AK, Li X, De Blas AL (2006) Synaptic and nonsynaptic localization of $GABA_A$ receptors containing the α5 subunit in the rat brain. J Comp Neurol 499:458-470.

Shimizu E, Tang YP, Rampon C, Tsien JZ (2000) NMDA receptor-dependent synaptic reinforcement as a crucial process for memory consolidation. Science 290:1170-1174.

Siegel E (2002) Mapping of the benzodiazepine recognition site on $GABA_A$ receptors. Curr Top Med Chem 2:833-839.

Sieghart W, Item C, Buchstaller A, Fuchs K, Hoger H, Adamiker D (1993) Evidence for the existence of differential O-glycosylated α5-subunits of the gamma-aminobutyric acid A receptor in the rat brain. J Neurochem 60:93-98.

Silva AJ, Kogan JH, Frankland PW, Kida S (1998) CREB and memory. Annu Rev Neurosci 21:127-148.

Smith SS, Gong QH, Li X, Moran MH, Bitran D, Frye CA, Hsu FC (1998) Withdrawal

from 3α-OH-5α-pregnan-20-one using a pseudopregnancy model alters the kinetics of hippocampal $GABA_A$-gated current and increases the $GABA_A$ receptor α4 subunit in association with increased anxiety. J Neurosci 18:5275-5284.

Smolders I, Lindekens H, Clinckers R, Meurs A, O'Neill MJ, Lodge D, Ebinger G, Michotte Y (2004) In vivo modulation of extracellular hippocampal glutamate and GABA levels and limbic seizures by group I and II metabotropic glutamate receptor ligands. J Neurochem 88:1068-1077.

Soghomonian JJ, Martin DL (1998) Two isoforms of glutamate decarboxylase: why? Trends Pharmacol Sci 19:500-505.

Sperk G, Schwarzer C, Tsunashima K, Fuchs K, Sieghart W (1997) $GABA_A$ receptor subunits in the rat hippocampus I: immunocytochemical distribution of 13 subunits. Neuroscience 80:987-1000.

Srinivasan S, Nichols CJ, Lawless GM, Olsen RW, Tobin AJ (1999) Two invariant tryptophans on the α1 subunit define domains necessary for $GABA_A$ receptor assembly. J Biol Chem 274:26633-26638.

Stephenson FA (2006) Structure and trafficking of NMDA and $GABA_A$ receptors. Biochem Soc Trans 34:877-881.

Sternfeld F, Carling RW, Jelley RA, Ladduwahetty T, Merchant KJ, Moore KW, Reeve AJ, Street LJ, O'Connor D, Sohal B, Atack JR, Cook S, Seabrook G, Wafford K, Tattersall FD, Collinson N, Dawson GR, Castro JL, MacLeod AM (2004) Selective, orally active gamma-aminobutyric acid A α5 receptor inverse agonists as cognition enhancers. J Med Chem 47:2176-2179.

Stroffekova K, Proenza C, Beam KG (2001) The protein-labeling reagent FlAsH-EDT_2 binds not only to CCXXCC motifs but also non-specifically to endogenous cysteine-rich proteins. Pflugers Arch 442:859-866.

Sur C, Fresu L, Howell O, McKernan RM, Atack JR (1999) Autoradiographic localization of α5 subunit-containing $GABA_A$ receptors in rat brain. Brain Res 822:265-270.

Sur C, Quirk K, Dewar D, Atack J, McKernan R (1998) Rat and human hippocampal α5 subunit-containing gamma-aminobutyric acid A receptors have α5β3γ2 pharmacological characteristics. Mol Pharmacol 54:928-933.

Tang YP, Shimizu E, Dube GR, Rampon C, Kerchner GA, Zhuo M, Liu G, Tsien JZ

(1999) Genetic enhancement of learning and memory in mice. Nature 401:63-69.

Taubenfeld SM, Wiig KA, Bear MF, Alberini CM (1999) A molecular correlate of memory and amnesia in the hippocampus. Nat Neurosci 2:309-310.

Taylor PM, Connolly CN, Kittler JT, Gorrie GH, Hosie A, Smart TG, Moss SJ (2000) Identification of residues within $GABA_A$ receptor α subunits that mediate specific assembly with receptor β subunits. J Neurosci 20:1297-1306.

Taylor PM, Thomas P, Gorrie GH, Connolly CN, Smart TG, Moss SJ (1999) Identification of amino acid residues within $GABA_A$ receptor β subunits that mediate both homomeric and heteromeric receptor expression. J Neurosci 19:6360-6371.

Teissere JA, Czajkowski C (2001) A β-strand in the γ2 subunit lines the benzodiazepine binding site of the $GABA_A$ receptor: structural rearrangements detected during channel gating. J Neurosci 21:4977-4986.

Thomas GM, Huganir RL (2004) MAPK cascade signalling and synaptic plasticity. Nat Rev Neurosci 5:173-183.

Thomas P, Mortensen M, Hosie AM, Smart TG (2005) Dynamic mobility of functional $GABA_A$ receptors at inhibitory synapses. Nat Neurosci 8:889-897.

Tretter V, Ehya N, Fuchs K, Sieghart W (1997) Stoichiometry and assembly of a recombinant $GABA_A$ receptor subtype. J Neurosci 17:2728-2737.

van Rijnsoever C, Tauber M, Choulli MK, Keist R, Rudolph U, Mohler H, Fritschy JM, Crestani F (2004) Requirement of α5-$GABA_A$ receptors for the development of tolerance to the sedative action of diazepam in mice. J Neurosci 24:6785-6790.

Vianna MR, Alonso M, Viola H, Quevedo J, de Paris F, Furman M, de Stein ML, Medina JH, Izquierdo I (2000) Role of hippocampal signaling pathways in long-term memory formation of a nonassociative learning task in the rat. Learn Mem 7:333-340.

Viola H, Furman M, Izquierdo LA, Alonso M, Barros DM, de Souza MM, Izquierdo I, Medina JH (2000) Phosphorylated cAMP response element-binding protein as a molecular marker of memory processing in rat hippocampus: effect of novelty. J Neurosci 20:RC112.

Wall MJ, Usowicz MM (1997) Development of action potential-dependent and independent spontaneous $GABA_A$ receptor-mediated currents in granule cells of

postnatal rat cerebellum. Eur J Neurosci 9:533-548.

Wang H, Olsen RW (2000) Binding of the $GABA_A$ receptor-associated protein (GABARAP) to microtubules and microfilaments suggests involvement of the cytoskeleton in GABARAP-$GABA_A$ receptor interaction. J Neurochem 75:644-655.

West AE, Griffith EC, Greenberg ME (2002) Regulation of transcription factors by neuronal activity. Nat Rev Neurosci 3:921-931.

Whiting PJ (2003) $GABA_A$ receptor subtypes in the brain: a paradigm for CNS drug discovery? Drug Discov Today 8:445-450.

Wieland HA, Luddens H, Seeburg PH (1992) A single histidine in $GABA_A$ receptors is essential for benzodiazepine agonist binding. J Biol Chem 267:1426-1429.

Wisden W, Laurie DJ, Monyer H, Seeburg PH (1992) The distribution of 13 $GABA_A$ receptor subunit mRNAs in the rat brain. I. Telencephalon, diencephalon, mesencephalon. J Neurosci 12:1040-1062.

Wisden W, Seeburg PH (1992) $GABA_A$ receptor channels: from subunits to functional entities. Curr Opin Neurobiol 2:263-269.

Wu GY, Deisseroth K, Tsien RW (2001) Activity-dependent CREB phosphorylation: convergence of a fast, sensitive calmodulin kinase pathway and a slow, less sensitive mitogen-activated protein kinase pathway. Proc Natl Acad Sci U S A 98:2808-2813.

Yee BK, Hauser J, Dolgov VV, Keist R, Mohler H, Rudolph U, Feldon J (2004) $GABA_A$ receptors containing the α5 subunit mediate the trace effect in aversive and appetitive conditioning and extinction of conditioned fear. Eur J Neurosci 20:1928-1936.

Zhang J, Campbell RE, Ting AY, Tsien RY (2002) Creating new fluorescent probes for cell biology. Nat Rev Mol Cell Biol 3:906-918.

10 Abbreviations

AMPA	α-amino-3-hydroxy-5-methylisoxazole-4-proprionic acid
BES buffer	N,N-bis(2-hydroxyethyl)-2-aminoethanesulfonic acid buffer
BIG2	brefeldin A-inhibited GDP/GTP exchange factor 2
CaMKII	calmodulin-dependent protein kinase II
CREB protein	cAMP responsive element binding protein
Cy3	Indocarbocyanine (absorption 550 nm, emission 570 nm)
Cy5	Indocarbocyanine (absorption 650 nm, emission 670 nm)
DIV	days in vitro
DMEM	Dulbecco's modified eagle medium
DMSO	dimethyl sulfoxide
E	embryonic day
EDT_2	1,2-ethanedithiol
EDTA	ethylenediamine tetraacetic acid
ELISA	enzyme-linked immunosorbent assay
ER	endoplasmatic reticulum
$GABA_A$ receptor	γ-aminobutyric acid type A receptor
GABARAP	$GABA_A$ receptor associated protein
GFP	green fluorescent protein
GODZ	Golgi-specific zinc finger protein
GRIF	$GABA_A$ receptor interacting factor
GRIP	glutamate receptor interacting protein
HAP1	Huntington-associated protein 1
HEK293 cells	human embryonic kidney cells
HeLa cells	immortalized human epithelial cells from cervical carcinoma
HRP	horseradish peroxydase

MAPK	mitogen-activated protein kinase
MEM	minimum essential medium
NGS	normal goat serum
NMDA	N-methyl-D-aspartate
NSF	N-ethylmaleimide-sensitive factor
p	phospho-
PBS	phosphate buffered saline
PCR	polymerase chain reaction
PFA	paraformaldehyde
PKC	protein kinase C
Plic-1	proteins that link integrin associated protein to the cytoskeleton
PRIP	phospholipase C-related inactive proteins
SAPK/JNK	stress-activated protein kinase/c-Jun NH_2 terminal kinase
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
Src	proto-oncogenic tyrosine kinase
TBST	tris buffered saline tween-20
TM	transmembrane
TMB	tetramethylbenzidine

11 Acknowledgements

I would like to thank Prof. Dr. Hanns Möhler for giving me the opportunity to perform this research project at the Institute of Pharmacology and Toxicology.

I am especially thankful to Dr. Dietmar Benke for guiding me through the PhD thesis and introducing me to the challenging "GABA world". I am grateful for his scientific support, personal attention and most of all: his patience.

I would like to thank Prof. Dr. Jean-Marc Fritschy for his interest in my project, scientific support and a good collaboration with his group. Special thanks for reviewing my PhD thesis and accepting to be my co-examiner.

Prof. Dr. Raimund Dutzler I thank for accepting to be my "Doktorvater" and for his interest in my progress during the past four years.

Many thanks to the actual and former members of the Neurobiochemistry Lab (Dr. Dietmar Benke, Thomas Grampp, Claudia Michel, Dr. Kathrin Sauter and all our semester and diploma students) for all the help, discussions and great working atmosphere.

Special thanks to Thomas Grampp for the excellent and creative technical help and most importantly for the good "lounge vibes" in the lab!

I would like to thank Dr. Barbara Lardi-Studler, Carolien Petitjean and Corinne Siedler for the introduction into neuronal cell culture, including all the tricks how to grow happy cells. Special thanks to Dr. Barbara Lardi-Studler for discussion and exchange regarding biarsenical labeling method.

Thanks to Dr. Detlef Boison and Cornelia Schwerdel for the help and collaboration on "glycine transporter project".

I would like to thank Dr. Anja Zeller for providing me with α5-GFP construct, Dr. Ivana Kotevic for GFP antibody, Thomas Bürli for helping with bungarotoxin labeling method and Ruth Keist for many good cloning advices. I am grateful to Dr. Edith Schneider Gasser and Dr. Shiva Tyagarajan on many fruitful and stimulating discussions.

Many thanks to Dr. Aree Soontornmalai, Dr. Nicolette Gauttier, Dr. Rafaela da Silva, Martina Baldinger & previous and current students and postdocs for the unforgettable time we spent in- and outside of the lab. It was a beginning of beautiful friendships!

Finally, I thank Rouven Bingel-Erlenmeyer for his huge support and understanding.

To my family and friends for their care and encouragement: thank you!

12 Curriculum vitae

Personal information

Name	Ela Balic
Date of birth	08.11.1977
Place of birth	Doboj, Bosnia-Herzegovina
Nationality	Croatian
Degree	Graduate Engineer of Biology, University of Zagreb, Croatia
Address	Bauherrenstrasse 32, 8049 Zurich, Switzerland
Telephone	+41-43-300 47 38
E-mail	elba@access.unizh.ch

Education

2003 – 2007	**Ph.D. Thesis at Pharmacology and Toxicology Institute, University of Zurich**
	Title: *Assembly, trafficking and downstream signaling of α5-$GABA_A$ receptors.*
	Supervised by:
	Dr. Dietmar Benke
	Prof. Hanns Möhler
	Prof. Jean-Marc Fritschy
	Prof. Raimund Dutzler
2000	**Diploma thesis in the Group of Prof. A. Plückthun, Institute of Biochemistry, University of Zurich**
	Title: *Generating Miniantibodies with Covalently Cross-linked Association Domains.*
	Supervised by:
	Prof. Andreas Plückthun
	Prof. Ivana Weygand
1999 – 2000	**Student exchange year at the University of Zurich**
1996 – 2001	**Biology studies at the Faculty of Science, University of Zagreb, Croatia**
	Degree: Graduate Engineer of Biology (major in molecular biology)
1992 - 1996	**Mathematics and Natural Sciences High School, Zagreb, Croatia**

Publication List

Kubetzko S, **Balic E**, Waibel R, Zangemeister-Wittke U, Pluckthun A (2006)

PEGylation and multimerization of the anti-p185HER-2 single chain Fv fragment 4D5: effects on tumor targeting.

J Biol Chem 281(46):35186-201.

Yee BK, **Balic E**, Singer P, Schwerdel C, Grampp T, Gabernet L, Knuesel I, Benke D, Feldon J,Mohler H, Boison D (2006)

Disruption of glycine transporter 1 restricted to forebrain neurons is associated with a procognitive and antipsychotic phenotypic profile.

J Neurosci 26(12):3169-81.

Selected Conferences

2006 **5th Forum of European Neuroscience, Vienna, July 2006**

Poster presentation:

Balic E, Rudolph U, Mohler H, Benke D

Impaired assembly of α5 subunit containing $GABA_A$ receptors in α5(H105R) knock-in mice.

2005 **A joint meeting of the Union of the Swiss Societies for Experimental Research, Swiss Society for Neuroscience, Swiss Society of Biological Psychiatry, Zurich, February 2005**

Poster presentation:

Balic E, Studler B, Mohler H, Benke D

Trafficking of $GABA_A$ receptors monitored via the biarsenical-tetracysteine in vivo labeling method.